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Effects of fibroblast growth factor 8 and 18 on ovine ovarian granulosa cell function

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Résumé

La famille des facteurs de croissance des fibroblastes (FGF) comprend 22 membres. La masse moléculaire des FGFs varie de 17 à 34 kDa. Ces facteurs partagent une homologie de séquence en acides aminés (13% à 71%) ainsi qu'une homologie de structure. Durant le développement embryonnaire, les FGFs ont des rôles variés comme la régulation de la prolifération, de la migration, et de la différenciation cellulaire, et le maintien de l'intégrité vasculaire. Plus spécifiquement, FGF8 et FGF18 ont des effets significatifs sur la modification de la fonction des cellules granulosa de l'ovaire ovine. Notre hypothèse est que malgré une grande homologie de séquence, FGF8 and FGF18 vont produire des effets distincts sur les cellules de la granulosa qui pourront être caractérisés par une modulation du protéome. L'objectif de cette étude était de quantifier et comparer différentiellement l'expression du protéome des cellules de la granulosa suite à une exposition à FGF8 et FGF18. Les cellules de la granulosa récoltées chez des brebis, indépendamment du stade du cycle œstral ont été, extraites et cultivées en utilisant un protocole standard. Les cellules ont ensuite été exposées à 10 ng/mL de FGF8 ou FGF18 pendant 30 minutes. Les protéines ont été extraites, les ponts cystéinés réduits et acétylés pour que finalement les protéines soient digérées avec la trypsine. En utilisant une méthode « bottom-up », les peptides trytiques ont été analysés par spectrométrie de masse permettant l'identification et la quantification des protéines. Les résultats ont démontré qu'il y avait une augmentation signification de l'expression de l'ensemble des protéines présentes dans les cellules de la granulosa suite à l'exposition de ceux-ci à FGF8 et FGF18. Cette observation est cohérente avec une croissance cellulaire menant à une augmentation de la prolifération. De plus, l'expression de plusieurs protéines impliquées dans des voies de signalisation associées aux FGFR incluant spécifiquement ATF1, STAT3, MAPK1, MAPK3, MAPK14, PLCG1, PLCG2, PKCA, PIK3CA, RAF1, GAB1 et BAG2 a augmenté significativement (>1.5 fois ; $p < 0.01$). Tacitement, ces résultats suggèrent une activation de la voie MAPK/ERK cohérente avec ce qui est connu dans la littérature et nos hypothèses. De plus l'expression de deux facteurs de transcription, STAT3 et ATF1 ont été fortement augmentés. Ils sont directement impliqués dans la croissance, la prolifération et les mécanismes de survie cellulaire. Cette augmentation très significative de STAT3 et ATF1 pourrait avoir des conséquences importantes sur la viabilité de l'ovocyte.

Mots-clés : Protéomique, spectrométrie de masse, l'ovaire, les cellules de la granulosa, La facteur de croissance des fibroblastes, protéines kinases, les facteurs de transcription, la prolifération

Abstract

Fibroblast Growth factors (FGFs) are growth factors which have diverse biological activities including broad mitogenic and cell survival activities. FGFs constitute a large family of 22 distinct polypeptide growth factors varying in size from 17 to 34 kDa and have between 13 to 71% sequence homology. More specifically, FGF8 and FGF18 are both important modulator of granulosa cell functions. FGF8 and FGF18 are homologous factors which possess similar sequence homology, but we hypothesized they may interact to FGFRs differently leading to distinct effects, particularly on granulosa cell growth and induce proliferation following a short period of exposition. This study was performed to investigate the effects of FGF8 and FGF18 on ovine granulosa cells proteome. Ovine ovaries were obtained from adult sheep's irrespective of stage of estrous cycle and were cultured using a standard protocol. Granulosa cells were harvested from follicles then, seeded and cultivated. After, they were exposed to 10 ng/mL of FGF8 or FGF18. Cell proteins were extracted, cysteine bonds were reduced and acetylated and proteins were digested with trypsin. Tryptic peptides were analyzed using a bottom-up proteomic approach, mass spectrometry and a label-free quantitation method. The results obtained revealed following treatment with FGF8 or FGF18 for 30 minutes, an important shift toward upregulation for the entire granulosa cell proteome was measured. Additionally, several proteins, including ATF1, STAT3, MAPK1, MAPK3, MAPK14, PLCG1, PLCG2, PKCA, PIK3CA, RAF1, GAB1 and BAG2 were significantly upregulated (> 1.5-fold; $p < 0.01$). Results are suggesting the activation of the MAPK/ERK pathway as expected. However, it is important to note that ATF1 and STAT3 are important transcription factors involved in cell growth, proliferation and survival and consequently can hamper or rescue the normal ovine reproductive system function. Both are strongly interacting with the MAPK/ERK pathway. They are directly involved in the growth, proliferation and mechanisms of cell survival. This very significant increase of STAT3 and ATF1 could have important consequences on the viability of the oocyte.

Keywords: Proteomics, mass spectrometry, ovary, granulosa cells, fibroblast growth factors, protein kinases, transcription factors, proliferation

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List of abbreviation

AMH: Anti-Mullerian hormone

AREG: Amphiregulin

ATF1: Accelerating transcription factor 1

BAG2: Bag family molecular chaperone regulator 2

BTC: Betacellulin

BMP: Bone morphogenetic protein

cAMP: cyclic Adenosine monophosphate

CALM4: Calmodulin

CDC25: Cell-division cycle 25

CHORDC1: Cysteine and histidine-rich domain-containing protein 1

CHOP: DNA-damage-inducible transcript 3(GADD153)

CGCs: Cumulus granulosa cells

COCs: Cumulus oocyte complexes

CREB: Cyclic AMP response element binding protein

CNS: Central nerves system

CYP11A1: Cytochrome p450 cholesterol side-chain cleavage

CYP17A1: cytochrome P450 17α-hydroxylase

DIA: Data independent analysis

D: Ig like domain

DF: Dominant follicle

DUSP: Dual specific phosphatase

E2: Estradiol

ECM: Extracellular matrix

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ELK: ETS-domain protein (SRF accessory protein)

ERK1/2: Extracellular regulating kinase ½

ER: Estrogen receptor

EREG: Epiregulin

FGFs: Fibroblast growth factors

FGFRs: Fibroblast growth factors receptors

FSH: Follicle stimulating hormone

FSHR: Follicle stimulating hormone receptor

GAB1: GRB2-associated binding protein 1

GCs: Granulosa cells

GDF: Growth and differentiation factor

GDNF: Glial cell- drive neurotrophic factor

GnRH: Gonadotropin releasing hormone

HCG: Human chorionic gonadotropin

HSD3B: 3β-Hydroxysteroid dehydrogenase

HSD17B: 17β-Hydroxysteroid dehydrogenase

HSP90: Heat shock protein 90

HSP27: Heat shock protein 27

HSPGs: Heparan sulfate proteoglycan

HRAM: High-resolution, and accurate-mass

IGF-1: Insulin like growth factor 1

IGFBP: Insulin growth factor binding protein

Ig: Immunoglobulin

IMM: Inner mitochondrial membrane

IOI: Interovulatory interval

LF: Largest follicle

LH: Luteinizing hormone

LHCGR: Luteinizing hormone choriogonadotropin receptor

MAPK: Mitogen-activated protein kinase

MGC: Mural granulosa cells

MSK: Mitogen and stress activated kinase

OMM: Outer mitochondrial membrane

P4: Progesterone

PGC: Primordial germ cells

PGF2γ: Prostaglandin F2 alpha

PLCG: Phospholipase c, gamma

PMSG: Pregnant mare serum gonadotropin

PI3K: Phosphatidylinositol 3-kinase

PKC: Protein kinase c

PTPases: Protein tyrosine phosphatase

RAF1: Rapidly accelerated fibrosarcoma

RAS: Rat sarcoma

rRNA: Ribosomal ribonucleic acid

RTKs: Tyrosine kinase receptors

stAR: Steroidogenic acute regulatory protein

STAT3: Signal transducers and activators of transcription

SRF: Serum response factor

TC: Theca cells

TGF β: Transforming growth factor β

tRNA: Transfer ribonucleic acid

VEGFRs: Vascular endothelial growth factor receptors

mTORC1: Mechanistic target of rapamycin complex 1

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Introduction

Fibroblast Growth factors (FGFs) are a large family of growth factors that signal through their binding to specific tyrosine kinase receptors. FGFs are proteins that have diverse biological functions such as cell proliferation, survival, migration, and differentiation in many organs. There are four gene family members that regulate a variety of developmental processes, including brain patterning, branching morphogenesis and limb development (Beenken and Mohammadi 2009). Genetic studies in humans and mice showed that mutations leading to disruption of FGF signaling cause a variety of developmental disorders including dominant skeletal diseases, infertility, and cancer (Cotton, O'Bryan et al. 2008). Several mitogenic, cytoprotectively, and angiogenic therapeutic applications of FGFs have been recently discovered. FGFs have an important role in organ development through Mesenchymal-epithelial signaling pathways. It is also essential for airway branching in the developing mouse lung. FGF18 causes alveolar development that acts through epithelial FGFR2c. FGF8 plays an important role in differentiation of the midbrain into cerebellum (Colvin, White et al. 2001, Usui, Shibayama et al. 2004).

Noticeably, these FGFs have a great impact on pre-natal organ development. However, the post-natal ovary with the resting pool of primordial follicles is a site of constant development. These follicles contain an immature oocyte surrounded by a layer of squamous epithelial called pre-granulosa cells. To progress into growing follicles, this cell layer develops into cuboidal (Knight and Glister 2006). Indeed, it proliferates and acquires a layer of mesenchymal theca cells. These cells along with epithelial cells proliferate and increase their communication to follicular antrum (Buratini Jr, Pinto et al. 2007). FGFs play important roles in regulating the initiation of primordial follicle growth, oocyte and follicle survival, granulosa and theca cell proliferation and differentiation, corpus luteum formation, steroidogenesis, angiogenesis and also FGFs constitute a group of well-known paracrine regulators within the ovarian follicle (Itoh and Ornitz 2004). FGFs are classified into subfamilies, and of interest to this thesis is the FGF8-subfamily that also includes FGF18. These two proteins have similar receptor activation patterns and it could be proposed that they could take similar actions in ovine granulosa cells. FGF8 is a mitogenic growth factor that increases follicular health by increasing proliferation and

suppressing cell differentiation. FGF18 expression pattern is a mesenchymal-epithelial signaling pathways (Buratini, Teixeira et al. 2005). The intracellular pathways used by FGF is binding to FGF receptor, activated FGF receptor (FGFR 1c,2c,3c,3b and FGFR4) dimerizes and autophosphorylation of specific tyrosine residues then two main branches are activated: phosphorylation of MAPK via phospholipase C and activation of phosphatidylinositol-3-kinase then AKT and protein kinase c (PKC) pathways. It is important to understand that this pathway is essential in granulosa cells (Dailey, Ambrosetti et al. 2005).

The objective of the present study was to compare and differentiate at the proteome level, the effect trigger by the activation of FGFRs (i.e. FGFR2 and FGFR3) by two ligands, FGF8 and FGF18, in ovine granulosa cell using a bottom-up proteomics, mass spectrometry, label-free quantitation and bioinformatics. The outcome of this study will help to foster a better understanding of the FGF signaling pathway outcomes and the consequences of cell fate.

CHAPTER 1:

LITERATURE REVIEW

1. The ovary

The ovaries are the female gonads, and they are found in pairs located in the pelvic area. They have two main functions in reproduction : The first is gametogenesis and the second is the secretion of female sexual hormones such as estrogens and progesterone that are required for follicular development, maintenance of estrous cyclicity and reproductive functions including preparation of the reproductive tract for fertilization and subsequent establishment of pregnancy (Marieb, Lachaîne et al. 1993).

Sheep ovaries are almond form in the abdominal cavity. The ovary produces the egg by a process called oogenesis (parker 1979). In contrast to the continuous production of sperm (spermatogenesis) in the male, oogenesis is cyclic. This cycle (called the estrous cycle) has a characteristic time length and consists of a definite sequence of events, both physiological and behavioral. The secretion of the ovarian hormones in turn is precisely regulated by the hypothalamic-pituitary axis. The complex interactions and regulations of the hypothalamic, pituitary, and ovarian hormones are collectively responsible for the regular and predictable ovulatory menstrual cycle and fertility in females (Parker 1979, Gupta and Chia 2013).

2. The follicle

The ovary contains several thousand structures called primary follicles. Each follicle consists of a germ cell surrounded by a layer of somatic cells. This germ cell has the potential to mature into an egg if the follicle completes development. However, most of the primary follicles never develop. Rather, they die and are replaced by newly formed primary follicles. The relatively few primary follicles that develop completely do so through a series of phases. Many layers of cells are added to the single layer of cells surrounding the egg in the primary follicle and, forming a central cavity (Parker and Macnair 1979).

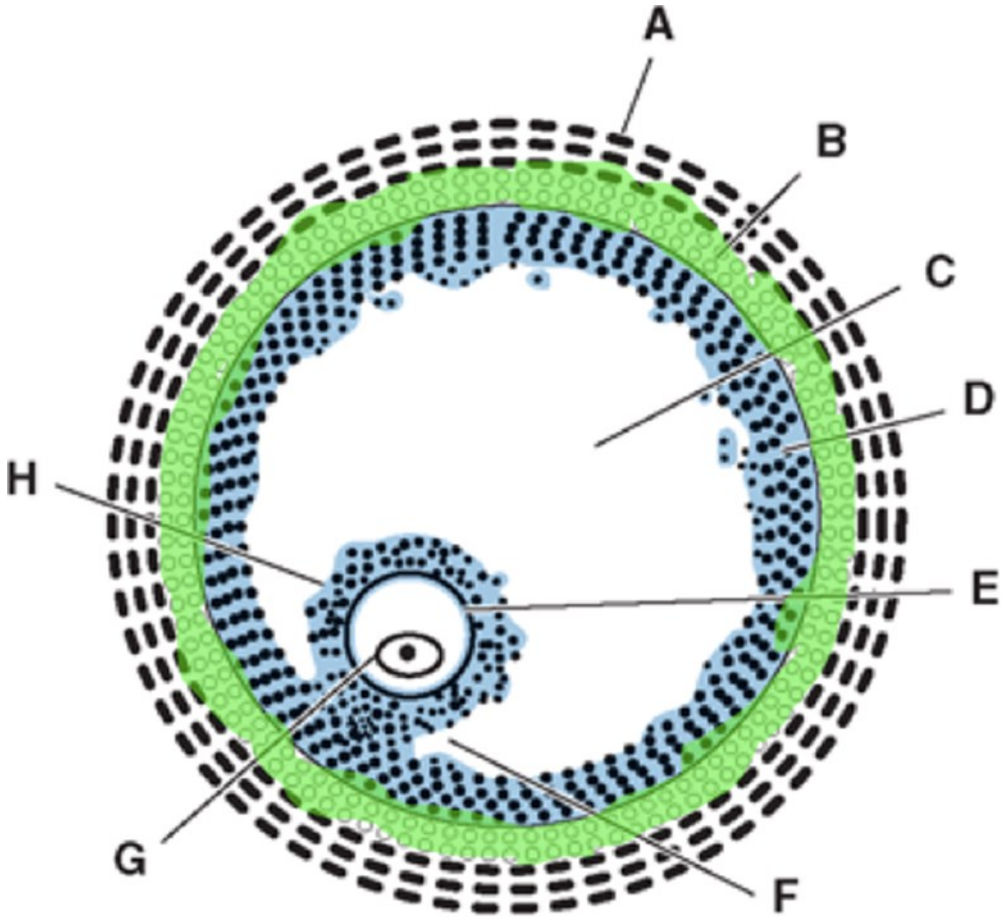
Reproductive efficiency can be a major determinant in ovulation rates. Recruitment processes and leads to the development one or several depending on species and breeders. After selection, ovulatory follicle (s) becomes dominant and progress for ovulation. This selection is co-ordinated by an endocrine and paracrine regulatory system involving several growth factors and other locally produced factors. The structure of the follicle changes during development and

can be classified into three different groups according to their size, complexity and responsiveness to circulating gonadotropins: preantral, antral and preovulatory follicles (McGee and Hsueh 2000, Hunter, Robinson et al. 2004).

2.1 Structure

Ovarian follicles are the basic units of female reproductive biology. Each of them contains a single oocyte surrounded by the zona pellucida and one or more layers of somatic cells referred to as cumulus granulosa cells (CGC). The antrum and the basal lamina which separates mural granulosa cells from the theca cells are of stromal origin and considered to be the interstitial tissue of the follicle (Gospodarowicz 1974).

Ovarien follicule structure



A-Theca externa
B-Theca interna
C-Antrum
D-Follicular fluid
E-Zona Pellucida
F-Cumulus granulosa cells
G-Oocyte
H- Corona radiata

Figure 1: Schematic representation of a pre-ovulatory structure mammalian follicle**.**
The cell types comprising the follicle are shown; the fully-grown oocyte and cumulus granulosa cells, Theca cells, extracellular matrix produced by the oocyte (zona pellucida) and antrum.
Source: Paulsen DF: histology and cell biology, Examination & Broad review/5th edition.
www.accessmedicine.com

2.1.1 Oocyte

The oocyte is the female germ cell in reproduction. In other words, it is an immature ovum or egg cell and it is produced in the ovary during female gametogenesis (Sigel and Minier 2005). Prior to fertilization; the number of oocytes in the mammalian ovary is fixed early in life. The development of the oocyte starts with the primordial germ cells (PGCs) which undergo meiosis to form an oogonium (Voronina and Wessel 2003). PGCs could perform extensive migration from the place of their formation to the developing gonad. This process is regulated by somatic germ cell interactions and some additional factors including FGFs (Sigel and Minier 2005, Takeuchi, Nakashima et al. 2005). The central role of the oocyte in the establishment of the fate of the embryo is indisputable (Coticchio, Sereni et al. 2004).

The best-documented stimulators of oocyte maturation are hormones and growth factors. Some mechanisms of maturation have been proposed and they include: 1) the production of a maturation-inducing substance by follicular cells that drives oocytes to mature, possibly involving activation of membrane receptors by steroid hormones; 2) inactivation of follicle-derived maturation inhibitors; and 3) inhibition of gap junction-mediated transport to prevent transfer of follicle-derived inhibitors (Corner, Hopkinson et al. 2005). The oocyte was considered only a passive recipient of developmental signals from oocyte-associated granulosa cells, communication between oocytes and granulosa cells is bidirectional (Eppig 2001).

2.1.2 Granulosa cells

In the primary follicle and during follicle development granulosa cells advance to form a multilayered cumulus oophorous surrounding the oocyte in the preovulatory or antral follicle. Granulosa cells generally produce sex steroids, and numerous growth factors that interact with the oocyte during its development. The sex steroid production consists of follicle-stimulating hormone (FSH) that stimulates granulosa cells to convert androgens produced by thecal cells to estradiol by aromatase during the follicular phase of the estrus cycle. However, after ovulation the granulosa cells turn into luteinized cells that produce progesterone. The progesterone may maintain pregnancy and causes production of a thick cervical mucus that inhibits sperm entry into the uterus (Veitch, Gittens et al. 2004). GC lack vascular supply, therefore they require

contact with their neighboring cells via gap junctions. These gap junctions contain different connexins such as connexin 32, 43 and 45. Connexin 43 has been studied in mouse where it has been observed from the onset of folliculogenesis just after birth and persists through ovulation (Ackert, Gittens et al. 2001).

GCs are important for oocyte maturation as they provide nutrients for oocyte development. As follicles grow and the antrum cavity formed, the GC form different subtypes: the cumulus granulosa cells (CGC), which are in direct contact with the oocyte and have a high rate of proliferation, low steroidogenic capacity, low LH receptor (LHR) expression and high levels of insulin growth factor I (IGF-1); and the mural granulosa cells (MGCs) which have a primarily endocrine function and support follicle growth. These cells undergo final differentiation to luteal cells after ovulation (Albertini, Combelles et al. 2001). During graafian follicle development, the granulosa cells sequentially develop specific membrane receptor sites for FSH and LH. In vivo studies on the mechanism of granulosa cell differentiation have established that FSH induces the appearance of the LH receptor sites in the granulosa cell. The FSH-induced increase in LH receptors is essential for preparing the graafian follicle for the pre-ovulatory surge of LH which initiates ovulation and subsequent luteinization of the granulosa cells (Erickson, Wang et al. 1979, Grøndahl, Borup et al. 2009).

2.1.3 Theca cells

Theca cells (TC) are endocrine cells that play an essential role in fertility by producing the androgen substrate required for ovarian estrogen biosynthesis. The hyperactivity of theca cells causes infertility due to hyperandrogenism and lack of estrogen (Magoffin 2005). The theca cells are divided into two layers, the theca interna and the theca externa. The theca interna is responsible for the production of androstenedione, and indirectly the production of 17β estradiol, also called E2, by supplying the neighboring granulosa cells with androstenedione that with the help of the enzyme aromatase, can be used as a substrate for this type of estradiol. FSH induces the granulosa cells regulating biosynthesis of aromatase, an enzyme responsible for the conversion of androgens produced by the theca interna into estradiol. Moreover, the theca

interna is highly vascular and possesses LH receptors, not FSH. The estradiol promotes the formation of LH receptors on granulosa cells, which also have FSH receptors (Magoffin 2005).

During development, many follicles undergo atresia, and the TC is often the final follicular cells type to die. For those follicles that ovulate, the TC then undergoes hormone-dependent differentiation into luteinized TC of the corpus luteum (Young and McNeilly 2010).Theca cells are highly differentiated with structural features typical of steroid-secreting cells including abundant mitochondria with vesicular cristae, a granular endoplasmic reticulum, and lipid vesicles (e.g. the mitochondria contain the first enzyme in the steroidogenic pathway, cholesterol side-chain cleavage cytochrome P450 (CYP11A1), and the endoplasmic reticulum contains the remaining enzymes necessary for the catalysis of all reactions leading to the formation of androgens. The lipid vesicles store cholesterol esters (e.g. precursor of steroid hormone biosynthesis) which are transported into the mitochondria by steroidogenic acute regulatory protein (Manna, Dyson et al. 2009).

2.2 Growth and development

Follicular development and growth can be driven by different regulators and involve complex interactions between the three main cell types within the follicle: 1) theca cells 2) granulosa cells and 3) oocyte (Scaramuzzi, Baird et al. 2011). The systemic endocrine regulation of folliculogenesis is related not only to the pituitary gonadotropins FSH and LH, but various locally produced hormones and growth factors. The oocyte has been confirmed as a major regulator of preantral and early antral follicular growth (McGee and Hsueh 2000). On the other hand, late steps of antral follicle development and growth involve gonadotropins and growth factors, especially the insulin-like growth factor (IGF) system (Fortune, Rivera et al. 2004). The ovarian cortex contains follicles at different developmental stages and can be classified as primordial, primary, secondary and antral follicles. The fate of each follicle is controlled by endocrine and paracrine factors (Picton 2001).

Folliculogenesis starts before birth in some mammalian species (e.g. cow, sheep and buffalo) or shortly after birth in others (e.g. mouse, rat and hamster). By this time, all germ cells in the ovaries are primary oocytes, and they remain in this stage until puberty, when at each

oestrus cycle selected follicle(s) develop and acquire the potential to ovulate. Even before birth, some oocytes will die by apoptosis (Picton 2001, Paulini, Silva et al. 2014). Primordial follicles are characterized by a single and flattened layer of granulosa cells. These follicles constitute the ovarian reserve from which follicles are engaged in development. Follicular growth takes place in only a small number of follicles each period and during which the differentiation and proliferation of granulosa cells and the enlargement of the oocyte occurs (Paulini, Silva et al. 2014). The duration of folliculogenesis from primordial follicle to ovulation is remarkably consistent between sheep and cattle at approximately 4-6 months of age, but is shorter in pig. In cattle the mature ovulatory follicles demonstrate high expression of aromatase in the granulosa cells, high concentrations of estradiol in the follicular fluid and induction of LH receptors on the granulosa cells (Hunter, Robinson et al. 2004).

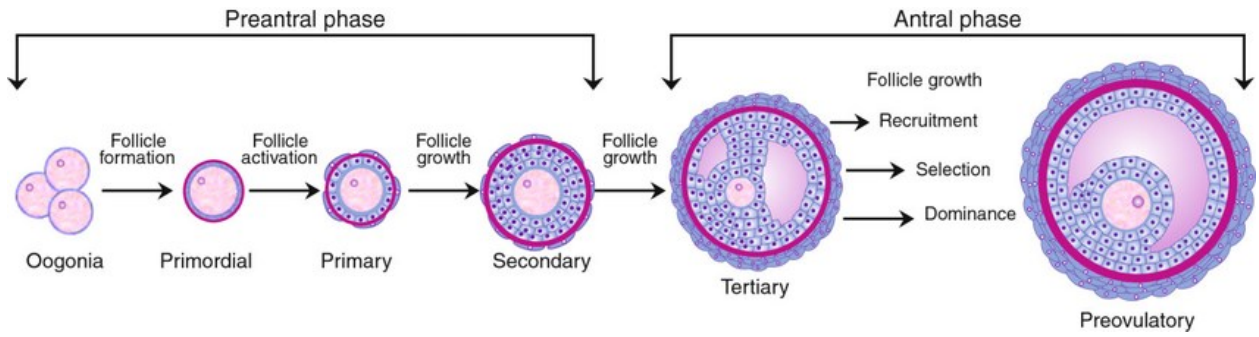


Figure 2: Schematic sequence of complete follicular development (Araújo, Gastal et al. 2014). Preantral phase: Formation and beginning of growth and activation of primordial follicles and growth of primary and secondary follicles. Antral phase: formation of tertiary follicle (antral-filled follicular fluid cavity). Follicle growth continues through the phases of recruitment, selection, dominance, and preovulatory stage of follicular waves. Oogonia develop from a primordial germ cell and differentiates into an oocyte in the ovary. Primordial follicle has a single layer of flattened granulosa cells. Primary follicle has a single layer of cuboidal granulosa cells. Secondary follicle has two or more layers of cuboidal granulosa cells and a small number of theca cells. All the preantral follicles have a primary oocyte. Tertiary follicle has several granulosa cell layers, theca cells, and primary oocyte and is characterized by an antral cavity which contains follicular fluid. Preovulatory or also called as Graafian follicle is the last stage of follicle development; these follicles are larger, have more antral fluid and may contain a secondary oocyte. Follicular fluid is a plasma exudate conditioned by secretory products from the granulosa cells and oocyte.

2.1.4 Recruitment

Follicle activation or recruitment takes place in two phases: 1) a continuous recruitment of the dormant primordial follicles into the growing follicle pool; and 2) a cyclical recruitment in response to FSH. Also, FSH can bind to GC of preantral follicles making them responsive to FSH and permitting them to follow a wave-like pattern in response to periodical endogenous surges of FSH (Adams, Jaiswal et al. 2008). Follicle recruitment is associated with initiation of simultaneous expression of cholesterol side-chain cleavage cytochrome P450 (CYP11A1) in GC of the recruited of follicles, which are likely to be increased by circulating FSH (Bao and Garverick 1998). Recruitment is not a random or isolated phenomenon. On the contrary, follicles seem to be recruited as groups or cohorts, suggesting that they have received a signal that allows them to continue growth and development rather than regress (Sisco, Hagemann et al. 2003).The signal that stimulates recruitment appears to be a slight elevation in plasma FSH. In cattle, not only does a secondary surge of FSH on the day of ovulation precede the first follicular wave of the cycle, but also slight elevations in FSH have been shown to precede the second and third follicular waves of the cycle and the waves that occur in prepubertal animal (Webb, Nicholas et al. 2002).

2.1.5 Selection

The number of follicles recruited is usually greater than the typical number of ovulatory follicles for a given species. In monovulatory species, selection is the process where a single follicle is chosen from the cohort of medium size growing follicle for further development while the rest become atretic (Fortune, Rivera et al. 2004). In polyovulatory species, multiple follicles are selected and grow synchronously until ovulation. The exact process of how a follicle is selected remains unknown, although it has been suggested that the selected follicle shows increased expression of FSH, LHCGR and 3β-HSD in GC, permitting them to be responsive to LH and continue developing in the face of lower FSH concentrations (Aerts and Bols 2010). It has been also proposed that the increased follicular growth rate is due to an increase in IGF1 bioavailability in the dominant follicle (Lucy 2007). It has been established that the development of one antral follicle until it becomes dominant requires 42 days in the cow, or the equivalent of two estrous cycles (Aerts and Bols 2010). Estradiol secretion decreases, as follicle growth slows

after ovulation due to reduction of aromatase activity from the granulosa cells. This decrease in negative feedback from the ovary, presumably allows the next small increase in basal FSH, which induces the next round of recruitment (Dunlop and Anderson 2014, Mesen and Young 2015). If the dominant follicle becomes the preovulatory follicle, a cascade of events started by the preovulatory LH surge results in ovulation. LH increases the synthesis of progesterone receptors, prostaglandins and epidermal growth factor (EGF)-like factor in GC, and induces the primary oocyte to complete meiosis I. There is also an up-regulation of the expression of proteases thought to play critical roles in follicular rupture (Russell and Robker 2007).

2.1.6 Atresia

During life, greater than 99% of the follicles present at birth are destined to degenerate. In humans ovary, less than 400 of more than 400,000 follicles found at puberty will eventually ovulate whereas the rest of the follicular population undergoes atresia (Tilly, Kowalski et al. 1991). Atresia happens at various stages of follicular development, but the collective evidence suggests that the stage of development where the follicle has to form an antrum and to maintain the granulosa-oocyte syncytium is the most critical time. The incidence of atresia in follicles is greater after antrum formation, just before the final stages of follicular development (Scaramuzzi, Baird et al. 2011). Both subordinate and dominant follicles may stop their growth and regress through the atresia process under different circumstances. Morphological signs of atresia include:1) decrease of follicle wall thickness characterized by the reduction of granulosa cells layer thickness, which become loose and disorganized (Irving-Rodgers, Van Wezel et al. 2001), 2) Cell degeneration, initially in the granulosa cells layer. The death of granulosa cells leads to almost total destruction of the granulosa cells layer lining the inner follicular wall with the consequent destruction of follicular structure (Yang and Rajamahendran 2000). Follicular atresia occurs by programmed cell death or apoptosis. Once initiated, this process is progressive and results in the disappearance of recognizable follicular structure. Preliminary studies in sheep injected with PMSG showed that exogenous gonadotropin may rescue atretic follicles in this species (Moor, HAY et al. 1978). Depletion by atresia of the ovarian follicular reserve starts during fetal life and continues throughout the reproductive life of the femelle.

2.2.4 Estrous cycle in sheep

Ewes are seasonally and intermittently polyestrous and classed as short-day breeders in contrast to long day breeders as the mare. They are sexually active and have regular estrous cycles only during the fall (short day light).

A complete estrous cycle includes development of an ovum on the ovary, a period of receptiveness to the ram (the heat or estrous period), ovulation and readying of the uterus for pregnancy during dioestrus with presence of progesterone produced by the corpus luteum. As ewes are polyestrous, there are multiple estrous cycles for the ewe to conceive during a single breeding season (Kennaway, Gilmore et al. 1982). The average estrous cycle (time from one ovulation to the next) is 17 days in the ewe. But because of environmental stressors, poor nutrition or severe weather (cold or heat), the estrous cycle can be disrupted. The length of the cycle (abnormally long or short) is also disrupted at the beginning or end of the breeding season (Wildeus 2000).

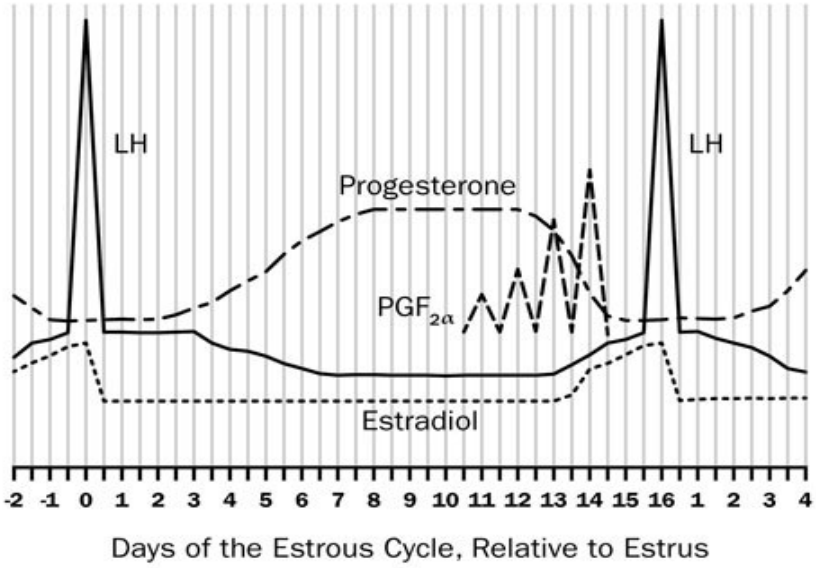


Figure 3. Estrous cycle. Sheep Production Handbook (2002).
The estrous cycle is controlled by a complex regulatory system involving several hormones. The hypothalamus sends gonadotropin-releasing hormone (GnRH) to the pituitary gland, which sends LH and FSH to the ovaries, prompting the ovaries to grow follicles. As the follicles grow, they create estradiol, which is fed back to the brain and causes the ewe to come into estrous. Once the increase of E2 synthesis within the follicle has begun, it has the capacity of self-augmenting by upregulating androgen synthesis in TC and pregnenolone in GC (Beg and Ginther 2006). The amount of estradiol being sent to the brain increases as the maturing follicles get larger. When the follicles reach 0.5-1 cm in diameter, blood concentration of estradiol peaks, and the brain releases a large amount of LH, which causes ovulation. After ovulation occurs, the follicle that the egg was in collapses and forms a corpus luteum, which secretes progesterone. This increase in progesterone tells the hypothalamus to decrease production of GnRH, resulting in reduced follicular growth, causing estrus and ovulation to be suppressed if the progesterone level is high. If a pregnancy is not established (which would keep the progesterone levels high), the uterus will secrete the hormone prostaglandin (PGF2α) This hormone causes the corpus luteum to die and decreases progesterone. As a result, the hypothalamus starts producing GnRH, and the cycle begins again (Kennedy 2012) (Lobato, Ferro et al. 2013).

Table 1: Reproductive Characteristics of ewes. Hafez, Reproduction in Farm Animals (2000). www.omafra.gov.on.ca

Length of cycle	17 days
Luteal phase	14 days
Follicular phase	3 days
Length of esterous	6 hours
Ovulations	1-2 oocyte
Cycles	Seasonally polyestrous- short days (fall)
Puberty	6 months
Gestations	147 days

3. Steroidogenesis

Steroidogenesis, is the process by which cholesterol is converted to steroid hormones. Reproduction of steroids is one of the most important functions of the follicle. In ruminants follicular steroidogenesis usually starts with cholesterol and ends with the formation of several steroid metabolites (Simpson and Davis 2001). This involves both Theca and granulosa cells. Basically, cholesterol is imported into the cell through internalization of blood-borne lipoproteins. Within the cell, cholesterol is maintained within lipid droplets as cholesterol esters. Cholesterol ester hydrolase enzyme converts the cholesterol esters to free cholesterol (Young and McNeilly 2010). Steroid hormones can be classified into five categories: glucocorticoids (cortisol), mineralocorticoids (aldosterone), androgens (testosterone), estrogens (estradiol and estrone) and progestins (progesterone). Steroidogenesis is limited to the adrenal cortex, testicular Leydig cells, ovarian granulosa and theca cells, and placental syncytiotrophoblast cells (Miller and Bose 2011).

The first step in steroidogenesis takes place within mitochondria, the mechanisms by which cholesterol is transported and loaded into the outer mitochondrial membrane (OMM). Steroidogenic acute regulatory protein (StAR), which is a member of a family of proteins that contain so-called START (StAR-related lipid transfer) domains, facilitates movement of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM). Steroidogenesis is initiated by mitochondrial cytochrome P450 cholesterol side-chain cleavage (CYP11a1) that cleaves the 20,22 bonds of insoluble cholesterol to produce soluble pregnenolone, and is the hormonally regulated, rate-limiting step in steroidogenesis. Pregnenolone may then be converted to progesterone by 3β-hydroxysteroid dehydrogenase (HSD 3β), which may be found both in the mitochondria and in the endoplasmic reticulum (ER). Alternatively, pregnenolone may be converted to 17α-hydroxy pregnenolone by the enzyme cytochrome P450 17α-hydroxylase (CYP17A1). The 17α-hydroxy pregnenolone can be converted to androstenedione by CYP17A1 and HSD3B. Androstenedione is then converted into testosterone by the 17β-hydroxysteroid dehydrogenase (HSD17B), TCs secrete androstenedione and testosterone and GCs can convert androstenedione to estradiol by HSD17B and testosterone to estrone by CYP19A1 (Miller 2011).

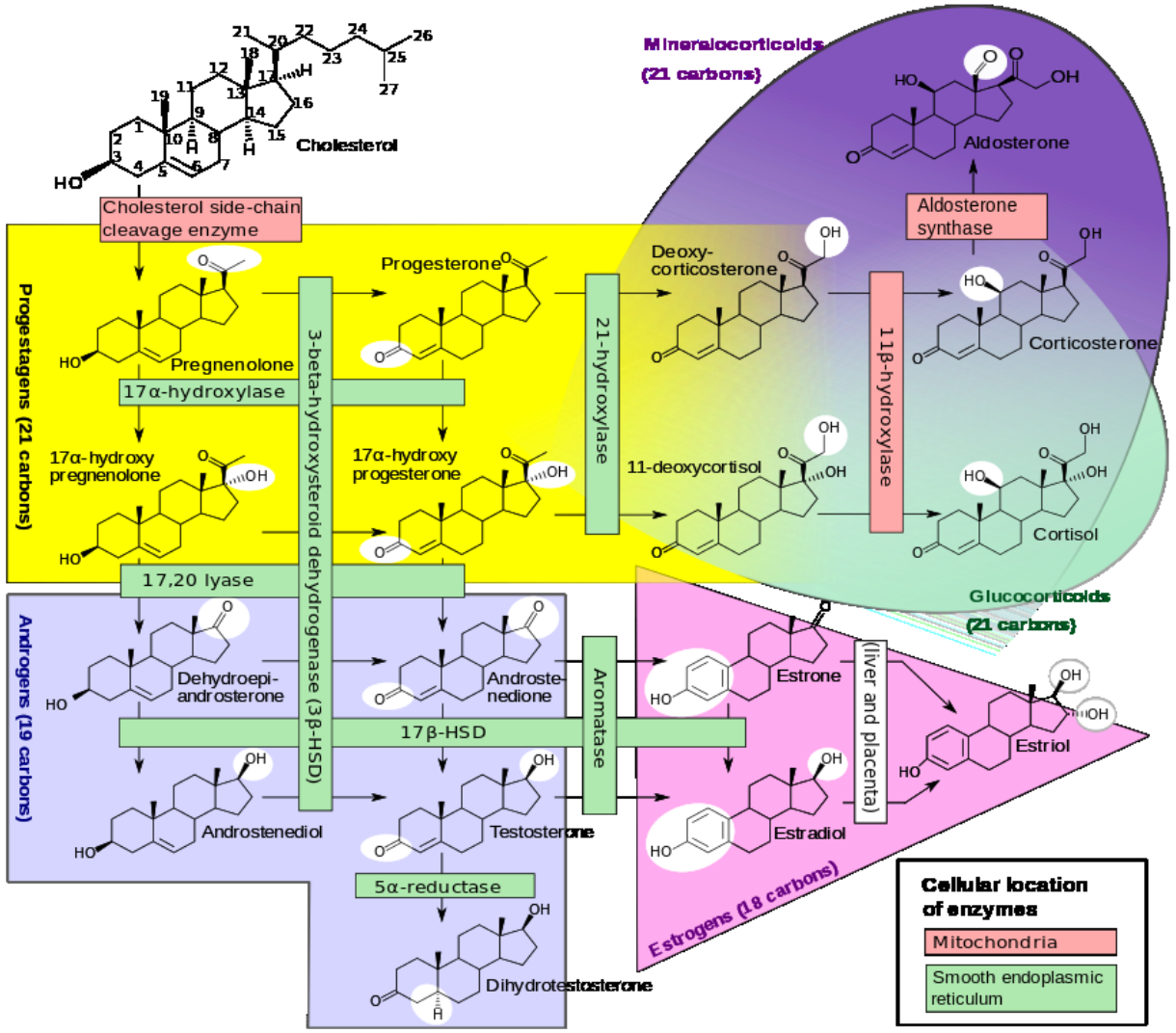


Figure 4. Steroidogenic pathway.(MED LIBES, Online Medical Library)(Borgeest, Greenfeld et al. 2002).The antral follicle is responsible for producing female sex steroid hormones, and it does so by the process known as steroidogenesis. Steroidogenesis requires both theca and granulosa cells. The pathway that converts pregnenolone to progesterone is in corpus luteum. Steroidogenic enzymes present in these cells are responsible for metabolizing cholesterol to 17-β estradiol and other necessary sex steroid hormones and in theca cell pregnenolone converts to androgens and subsequently estrogens. EDCs can alter the expression, protein level, or enzyme activity of steroidogenic enzymes, thus resulting in altered sex steroid hormone.

3.1 Estradiol

Estradiol is the most important hormone during a female's reproductive season, and is required for reproductive and sexual function (Somjen, Kohen et al. 2004). Estradiol is produced by the ovaries, adrenal gland and the placenta during pregnancy. One characteristic of the growing follicle is its considerable capacity for E2 production. In cattle, E2 promotes development of preantral follicles and stimulates steroidogenesis. Shortly before the beginning of deviation between the largest follicle and the second largest follicle, there is a marked difference in concentrations of E2 in the follicular fluid of the two follicles. The E2 content is a key characteristic of a dominant follicle. In addition, E2 concentrations decrease in subordinate follicles while the dominant follicle continues growing. As the rate of growth of the follicle slows, estradiol concentrations do not decrease until the follicle starts to regress. All these make E2 a marker for health or atresia of follicles (Goebel, Birge et al. 1995).

3.2 Progesterone

Progesterone (P4) is a steroid hormone involved in pregnancy and embryogenesis. P4 is produced in TC and GC. During the beginning of follicular growth there are no differences in P4 levels between the two largest follicles. However, some studies have found that after the second largest follicle (subordinate follicle) starts regressing, there is an increase in P4, making unclear the role of progesterone in the process of growth and differentiation (Beg and Ginther 2006).The role of P4 is essential not only for the establishment but also for the maintenance of pregnancy , as it supports ovulation and uterine and mammary gland development (Yates, Li et al. 2010). The major source of P4 during early pregnancy is the corpus luteum, however, in some species like the ovine, the placenta produces a significant amount of P4 after 50 days of pregnancy. The genomic actions of P4 are mediated by the intracellular progesterone receptors, and blocking P4 binding sites results in abortion (Arck, Hansen et al. 2007).

4. Growth factors

Growth factors are proteins that bind to receptors present on the cell surface activating cellular proliferation, healing and/or differentiation (Rothe and Falanga 1989). Several growth factors are non-specific, stimulating cellular division in several different cell types; while others are specific to a particular cell-type. Growth factors are an important class of proteins playing a central role as signaling molecules between cells (Baird and Walicke 1989). Ovarian folliculogenesis is modulated by diverse growth factors, including insulin-like growth factors (IGFs), epidermal growth factors (EGFs), transforming growth factor β (TGF-β) and fibroblast growth factors (FGFs) (Ben-Ami, Schwaber et al. 2006).

4.1 Fibroblast growth factors (FGFs)

The fibroblast Growth factors (FGFs) are polypeptide growth factors which have diverse biological activities. They are proteins that regulate cell proliferation, migration, and differentiation in many organs (Itoh and Ornitz 2004). FGFs were found first in pituitary extracts by Armelin in 1973 when he was able to extract it from the cow brain (Armelin 1973). FGFs/FGFRs, and signaling cascades have been implicated in a diverse cellular processes such as proliferation, apoptosis, cell survival, chemotaxis, cell adhesion, motility, and differentiation (Itoh and Ornitz 2004). Genetic studies in humans and mouse showed that mutations leading to disruption of FGF signaling cause a variety of developmental disorders including dominant skeletal diseases, infertility, and cancer (Cotton, O'Bryan et al. 2008).

FGFs are secreted glycoproteins that are generally readily sequestered to the extracellular matrix, as well as the cell surface, by heparin sulfate proteoglycans (HPSGs). FGFs regulate a variety of developmental processes, including brain patterning, branching morphogenesis and limb development (Min, Danilenko et al. 1998). Several mitogenic, cytoprotective and angiogenic therapeutic applications of FGFs have recently been discovered. They have some pharmacological potential for instance, FGF19 has a crucial role in endocrine-acting bile acid, glucose and phosphate homeostasis (Beenken and Mohammadi 2009).

4.1.1 FGF Families

All FGFs have a high degree of amino acid sequence homology. However in mammals, FGFs can be classified in 3 different categories: canonical, hormone-like and intracellular FGFs. The fibroblast growth factor (FGF) family consists of 22 genes in the human genome. It was demonstrated that from these 22 genes, there are 18 that encode growth factors to function through activation of the FGF receptors. They are composed of five distinct receptors (i.e. FGFR1-FGFR5). The FGF members are numbered FGF1–FGF10 and FGF16–FGF23. Also, these 18 FGF proteins are classified into seven families based upon differences in sequence homology (Itoh and Ornitz 2004).

Table 2. Human FGF gene families. Members of each subfamily share increased sequence similarity and biochemical and developmental properties (Itoh and Ornitz 2004).

Family	**Subfamily**
FGF1	FGFs 1 and 2
FGF4	FGFs 4, 5 and 6
FGF7	FGFs 3, 7, 10 and 22
FGF8	FGFs 8, 17 and 18
FGF9	FGFs 9, 16 and 20
FGF11	FGFs 11, 12, 13 and 14
FGF 19	FGFs 19, 21 and 23

Twenty-two FGFs and four FGFRs were identified in humans and mice (Itoh and Ornitz 2004). Each FGFR is bound to a unique subset of FGFs. The specificity of each FGFRs is further regulated by splicing of their encoding FGFR genes (Qiang, Fanghong et al. 2006). The FGF signaling system acquires its functional diversity in both developmental and physiological processes through expansion of its diversified encoding genes (Itoh and Ornitz 2004, Liao,

Bodmer et al. 2009). Most FGFs include FGF8 subfamily (FGFs 3-8, 10, 17-19, 21 and 23) which have a N-terminal signal thet can be released from damaged cells or by an exocytotic mechanism that is independent of the endoplasmic reticulum and Golgi system. FGFs 11-14 are also deficient in signal peptides and thus remain intracellular and function within cells in a receptor-independent manner. By contrast, FGFs 9, 16 and 20 lack cleavable hydrophobic N-terminal sequence which causes them not to be secreted by the cells. FGF19 is the human ortholog of mouse FGF15. Despite having a N-terminal signal peptide, FGF22 remains attached to the cell surface rather than being secreted (Itoh and Ornitz 2004).

Table 3. Human (FGF1–14, 16–23) gene, protein, function, localization and therapeutic application

Gene	Protein	Receptors	Function,localizatio and therapeutic application
Fgf1	acidic FGF, a FGF	FGFR1,3b&3c;FGFR2,3b&3c FGFR3,3b&3c; FGFR4	Mitogenic effect. Released from damage cells.theraputic application is cardiovascular disease (Berisha,et al.2004).
Fgf2	Basic FGF, β FGF	FGFR1b,1c,2c,3c ,4	Skeletal, neuronal development. Released from damage cells and is used for cardiovascular disease and cancer (kuhn,willenberg et al.2012).
Fgf3	FGF3, int-2	FGFR1,3b;FGFR2,3b	Inner ear, skeletal development. Has amino terminal signal peptide and Secreted from cells. Therapeutic application is not stablished yet (Chaves, de Matos et al.2012).
Fgf4	Kaposi FGF,k FGF,hst-1	FGFR1c,2c,3c ,4	Angiogenesiss, cell mass proliferation. Secreted from cells. Therapeutic application is in gene transfer-mediated angiogenic therapy (Itoh and Ornitz 2004).
Fgf5	FGF5	FGFR1c,2c	Hair growth.Secreted from cells.Therapeutic application is unknown (Reuss, Dono et al. 2003).
Fgf6	Hst-2	FGFR1,3c;FGFR2,3c	muscle regeneration. secreted from cells. therapeutic application is not stablished yet. (Fiore, Sébille et al. 2000).
Fgf7	KGF	FGFR 2,3b	Hair follicle growth, ureteric bud growth. secreted from cells and therapeutic application is oral mucositis (Ohuchi, Hori et al. 2000).
Fgf8	AIGF	FGFR1,FGFR2,3c;FGFR3,3c,FGFR4	Gastrulation defect, CNS and limb development. Secreted from cells. Therapeutic application is organogenesis (Crossley, Martinez et al. 1996).
Fgf9	GAF	FGFR2,3c,FGFR3,3b,3c,FGFR4	Lung mesenchyme, XY sex reversal. Nevertheless secreted. Therapeutic application is unknown. (Kim, Kobayashi et al. 2006).
Fgf10	KGF-2	FGFR1,3b,FGFR2,3b	Development of multiple organs, including limb, lung, thymus, pituitary. Secreted from cells. Similar structure and function to FGF7 (Ohuchi, Hori et al. 2000).

Fgf11- Fgf14	iFGFs	unknown	Neuromuscular and Neurological development. Remain intracellular and they are not secreted and function within cells in recepto-independent manner (Itoh and Ornitz 2008).
Fgf16	FGF16	FGFR2,3c,FGFR3,3b,3c,FGFR4	Cerebellar and brown adipose tissue development. Similar to FGF9 Nevertheless secreted. Therapeutic application is not stablished yet (Miyake, Konishi et al. 1998)
Fgf17	X-FGF20	FGFR1,FGFR2,3c;FGFR3,3c,FGFR4	Developing brain and limb and expressed during gasterulation. Secreted from cells.Therapeutic application is not stablished yet (Cholfin and Rubenstein 2008).
Fgf18	FGF18	FGFR1,FGFR2,3c;FGFR3,3c,FGFR4	Skeletal and gonadal development and sex differentiation, tissue repair, tumor growth. secreted from cells. Have signal sequence. Therapeutic application is osteoarthritis, cartilage (Buratini et al ,2005)
Fgf19	hFGFs	FGFR1,FGFR4	Function as hormones. Secreted from cells. Therapeutic application is unknown (Kurosu, Choi et al. 2007)
Fgf20	X-FGF20	FGFR2,FGFR3c,3b	Embryonic development. Lack of N-terminal sequence not secreted by cells. It Regulate bile acid, fatty acid, glocose and phosphate metabolism in target cells (Ohmachi, Watanabe et al. 2000).
Fgf21	hFGFs	FGFR1c,2c,3c,4	Stimulate glucose uptake in adipocyte. secreted from cells. Therapeutic application is for diabetes (Nishimura, Nakatake et al. 2000).
Fgf22	FGF22	FGFR1,3b,FGFR2,3b	Hair development.secreted from cells.Therapeutic application is unknown. (Nakatake, Hoshikawa et al. 2001).
Fgf23	FGF23	FGFR1,3c	Acts as hormone that drives from bone and regulates kidney functions. Therapeutic application is for Hypophosphatemia (Urakawa, Yamazaki et al. 2006).

4.2.2 FGF Receptors

FGF receptors (FGFRs) contain 3 demains: an extracellular ligand-binding domain, a transmembrane domain and a split intracellular tyrosine kinase domain. The extracellular region contains two or three immunoglobulin (Ig)-like domains, and a heparin-binding domain.When FGFs bind to their specific FGFRs they induce dimerization and the phosphorylation of specific cytoplasmic tyrosine residues. Also after the activation of FGF receptors, several signal-transducing proteins become tyrosine-phosphorylated. Thus the phosphorylation of FGFRs triggres the activation of cytoplasmic signal transduction pathways (Itoh and Ornitz 2004).

In ovary, FGFs are predominantly expressed in theca cells**,** however, granulosa cells mostly express FGF receptors. FGFs cause, primordial follicle growth, oocyte and follicle survival, steroidogenesis and corpus luteum formation.

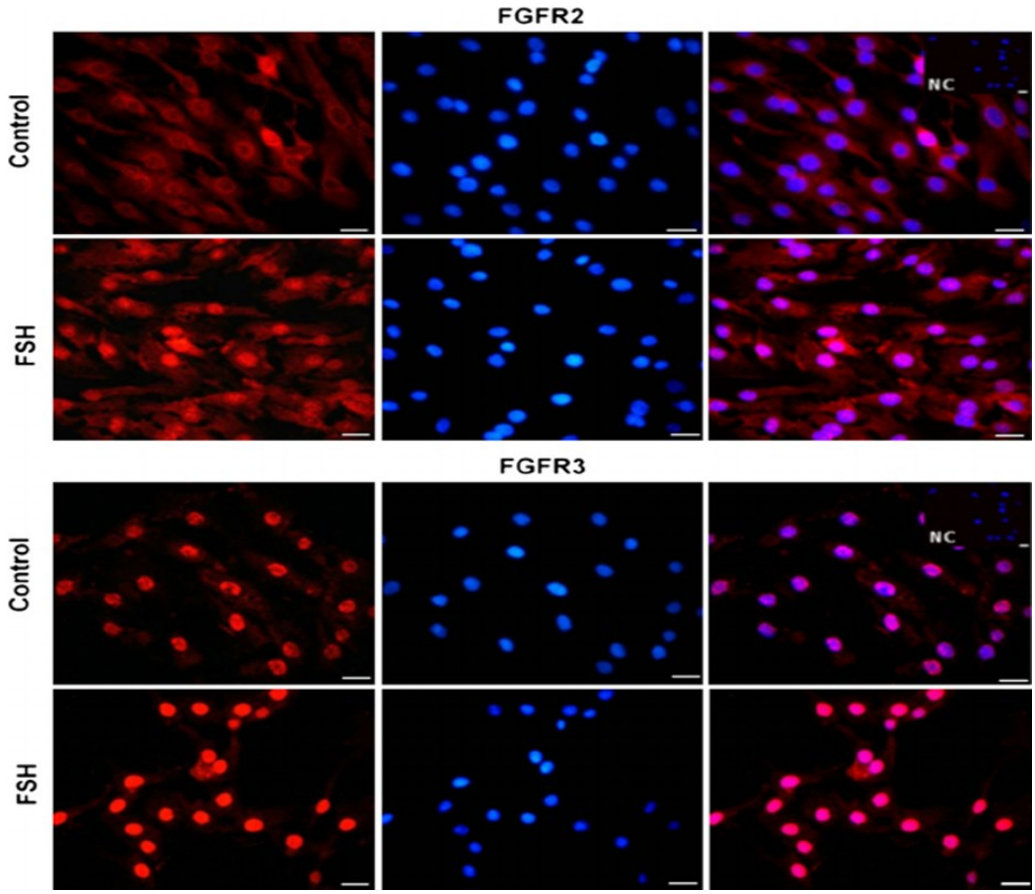


Figure 5: Localization and differential expression of the fibroblast growth factor receptor (Fatima, Evangelista et al. 2013).Immunofluorescence localization of FGFR2 and FGFR3 in luteinized granulosa cells on day 8 of the cell culture. The FSH-treated cells exhibited higher immunostaining of FGFR2 and FGFR3 in the nucleus than untreated cells. Scale bars = 50μm. 4,6-Diamidino-2-phenylindole was used as the nuclear-specific marker. FGFR2, fibroblast growth factor receptor 2; FGFR3, fibroblast growth factor receptor 3.

4.2.3 FGFR Structure

Control of FGF–FGFR specificity is mediated by the tissue-specific expression of particular ligands and receptors, coupled with several cell surface or secreted proteins that facilitate the FGF–FGFR interaction, such as the Klotho family for hormonal FGFs, which further increases ligand specificity (Itoh and Ornitz 2004, Chaves, de Matos et al. 2012).

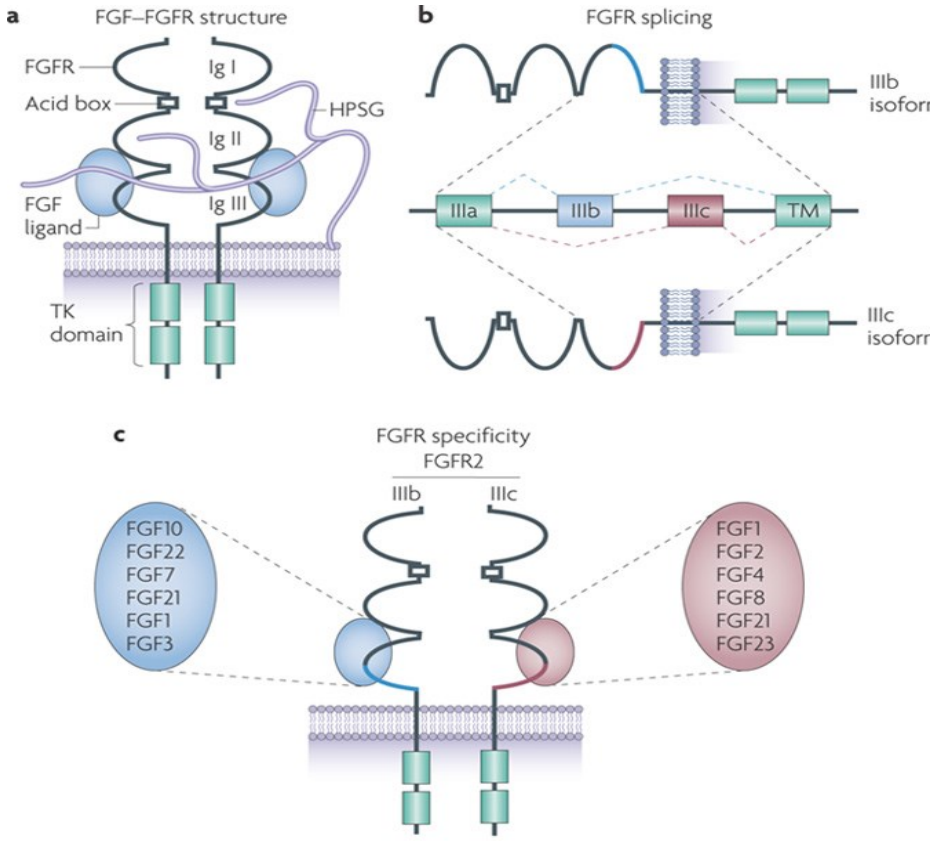


Figure 6. FGFR structure (Itoh and Ornitz 2004) (Beenken and Mohammadi 2009**).** FGFR1 to 4 have intracellular tyrosine-kinase domain, a single transmembrane domain extracellular part consisting of three Ig-like domain (D1,D2,D3) which are responsible for the interaction and specificity with FGFs. FGFs bind and activate tyrosin kinase receptors in an HSGAG-dependent manner. FGFR1-R4 encode receptors consisting of three extracellular immunoglobulin domains (D1-D3). They have a serine-rich sequence in the linker between D1 and D2, termed the acid box. The D2-D3 fragment of the FGFR ectodomain is necessary for ligand binding and specificity. The D1 and acid box are proposed to have a role in receptor autoinhibition. several FGFR isoforms called exon skipping remove D1 domain and/or acid box in FGFR1-R3. Alternating splicing in the second half of the D3 domain of FGFR1-3 yiels b (FGFR1b-3b) and c (FGFR1c-3c) isoforms that have distinct FGF specific bindings and are predominantly epithelial and mesenchymal, respectively. Except for FGF1 which activates both splice isoforms the FGF1-3 bind to either epithelial or mesenchymal FGFRs.

4.3.3 FGF signaling pathways

Fibroblast growth factor receptor (FGFR) belongs to the family of receptor tyrosine kinases (RTKs). Binding of fibroblast growth factor (FGF) to its receptor induces receptor dimerization and tyrosine phosphorylation. The phosphorylated form of the receptor can initiate several signal transducing pathways depending of cell specificity (Turner and Grose 2010). This is a unique mechanism for generation of signal diversity whereby coordinated assembly of a multi-docking protein complex can activate different downstream signaling pathways. (Cao, Cao et al. 2008).

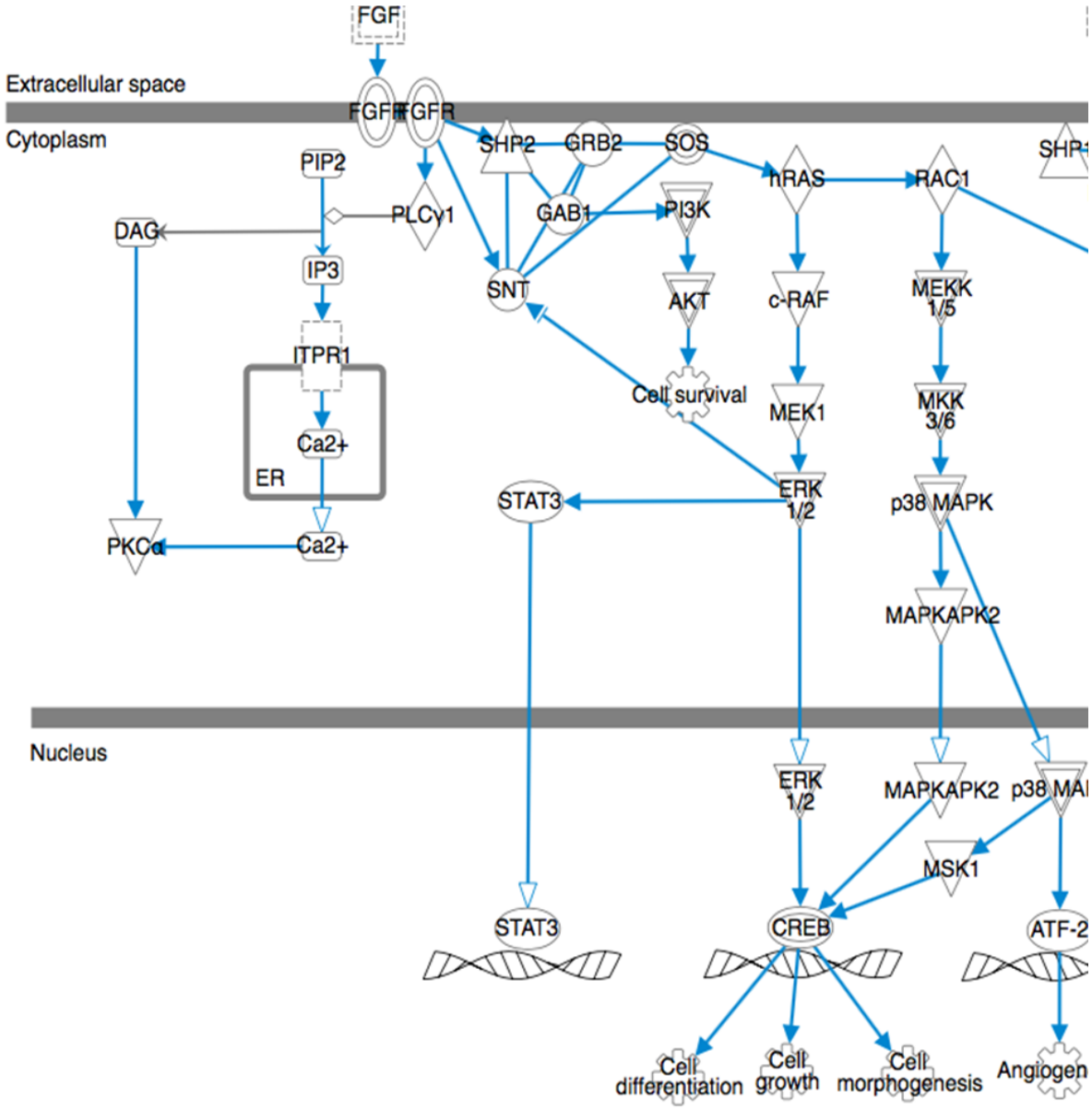


Figure 7: FGF signalling pathways.(targetexplorer.ingenuity.com) (Turner and Grose 2010). Binding of fibroblast growth factor (FGF) to its receptor induces receptor dimerization and tyrosine phosphorylation. The phosphorylated form of the receptor can initiate several signal transducing pathways depending of cell specificity. FGFR phosphorylation causes the tyrosine phosphorylation of SNT1 which leads to GRB2-mediated complex formation with docking protein GAB and nucleotide exchange factor SOS. These key events then lead to the activation of one of several pathways: the Ras/Raf/MEK/ERK pathway, the Rac1/MEKK/p38MAPK pathway or the PI3K/AKT pathway. Finally, transcriptional regulators like CREB, STAT3 and ATF-2 are activated leading to various cellular processes like cell growth, differentiation and angiogenesis. FGFR is also able to directly activate PLCγ and consequently cause the activation of protein kinase C and increased intracellular Ca^{+2}.

4.4.4 FGF roles and functions

FGFs have potential effects on the regeneration of tissues (Moya, Cheng et al. 2010).They also play fundamental biological functions observed in vitro and in vivo, including roles in mitogenesis, cellular migration and differentiation, angiogenesis and wound healing (Itoh and Ornitz 2004). FGFs were utilized for the regeneration of damaged tissues, including skin, blood vessel, muscle, adipose, tendon/ligament, cartilage, bone, tooth, and nerve (Freudenberg, Hermann et al. 2009). The expression patterns of FGFs suggest that they have an imortant role in development and progression of various malignant diseases. FGF2 exerts mitogenic effects and is over-expressed in human tumor cell lines. However FGF2 expression may also be associated with prognosis in ovarian and breast cancer. FGF1 can display biological activities similar to those of FGF2 (Le Page, Ouellet et al. 2006).

The 22 members of the mammalian FGF family are differentially expressed in many tissues, but the patterns and timing of expression are specific (Golub, Adelman et al. 2000). The subfamilies of FGFs also have similar patterns of expression. Some FGFs are expressed particularly during embryonic development (i.e. FGF3,4,8,15,17 and 19), whereas others are expressed in embryonic and adult tissues (i.e. FGF1, 2, 5-7, 9-14, 16, 18 and 20-23) (Ornitz and Itoh 2001). FGF signaling extends too many physiological roles in the adult organism, including the regulation of angiogenesis and wound repair. Some growth factors were identified for regulating the initiation of primordial follicle growth, granulosa and theca cell proliferation and differentiation, angiogenesis and steroidogenes. (Chaves, de Matos et al. 2012). FGF8 at first was detected and expressed in oocytes of small and large antral follicles of adult mice ovaries. FGF8 and FGF18 are key mesenchymal-epithelial signaling molecules in a variety of tissues, especially during organogenesis. Follicle growth involves communication between theca cells and granulosa cells, as well as between granulosa/cumulus cells and the oocyte. Theca cells are of mesenchymal origin and are known to express a number FGFs, including FGF1, FGF2, FGF7, FGF10 and FGF18. It has been previously demonstrated that FGF8 and FGF18, modulate ovarian function. They activate the same receptors, and the typical response to FGF is increased proliferation (Hawkins and Matzuk 2010). Degradation of FGF8 by loss-of-function leads to kallmann's syndrome (KAL1), a developmental disorder characterized by anosmia and hypogonadism (Falardeau, Chung et al. 2008).

4.1 Other growth factors involved in ovarian folliculogenesis

4.2.1 Insulin-like growth factors (IGFs) roles and functions

The IGF family includes two ligands, six binding proteins and two receptors. They are produced in ovarian follicles (Albertini, Combelles et al. 2001). Growth factors such as IGF-I and IGF-II, originating from endocrine sources or follicles are required for all phases of preovulatory growth and luteinization. Increased IGF-I plasma concentration leads to increasing the percentage of oocytes that matured to metaphase II in vitro. insulin like growth factor_ binding protein 1 (IGFBP-1 to -6) modulate the interaction of IGFs-I and –II with their receptors and each IGFBP has a distinct pattern of tissue distribution and regulation. However, in bovine antral follicles up to 9 mm, expressions of IGFBP-2 and IGFBP-4 mRNA are restricted to granulosa and theca cells, respectively (Beg and Ginther, 2006). IGFs are key intraovarian regulators of follicle growth, selection, atresia, cellular differentiation, and steroidogenesis, oocyte maturation, and cumulus expansion. Some of these actions are synergistic with gonadotropins, although most are not sustainable with IGFs alone and require gonadotropin actions (Kwintkiewicz and Giudice 2009). IGFs act synergistically with other mitogenic growth factors and steroids and antagonize the effect of antiproliferative molecules on cancer growth (Borinstein, Barkauskas et al. 2011). High levels of circulating IGF-I and low levels of IGFBP-3 are associated with increased risk of several common cancers, including prostate, breast, colorectum, and lung (Yu and Rohan 2000).

4.2.2 Epidermal growth factors (EGFs) roles and functions

The EGF is a protein of 53 amino acids which plays a crucial role in reproduction. Other members of the EGF family include TGF-α, amphiregulin(Areg), epiregulin(Ereg) , betacellulin(BTC), epigen, neuregulins and heparin-binding EGF-like growth factor. These proteins can work through four types of transmembrane receptors (Ben-Ami, Schwaber et al. 2006). The main functions of EGFR within the follicle are stimulating proliferation of GC, increasing P4 secretion, and controlling of the release of E2. In oocytes, EGF affects maturation and cumulus expansion, and inhibits apoptosis. The role of EGF has also been investigated as a paracrine mediator of LH induced ovulation (Quirk, Cowan et al. 2004). The EGF receptors also play important roles in cell proliferation, survival, adhesion, motility, invasion and angiogenesis

in normal and malignant cells, including ovarian tumors (Jiang, Grenley et al. 2011). EGF has 22 members of the mammalian FGF family are differentially expressed in many tissues, but the patterns and timing of expression is modified (Golub, Adelman et al. 2000). The subfamilies of FGFs also have similar patterns of expression. Some FGFs are expressed particularly during embryonic development such as (FGF3,4,8,15,17 and 19), whereas others are expressed in embryonic and adult tissues such as FGF1, 2, 5-7, 9-14, 16, 18 and 20-23 (Ornitz and Itoh 2001). FGF signaling extends too many physiological roles in the adult organism, including the regulation of angiogenesis and wound repair. Some growth factors have been identified for regulating the initiation of primordial follicle growth, granulosa and theca cell proliferation and differentiation, angiogenesis and steroidogenes. (Chaves, de Matos et al. 2012). FGF8 at first was detected and expressed in oocytes of small and large antral follicles of adult mice ovaries. FGF8 and FGF18 are key mesenchymal-epithelial signaling molecules in a variety of tissues, especially during organogenesis. Follicle growth involves communication between theca cells and granulosa cells, as well as between granulosa/cumulus cells and the oocyte. Theca cells are of mesenchymal origin and are known to express a number FGFS, including FGF1, FGF2, FGF7, FGF10 and FGF18. FGF8 and FGF18, modulate ovarian function. They activate the same receptors, and the typical response to FGF is increased proliferation (Hawkins and Matzuk 2010). Degradation of FGF8 by loss-of-function leads to kallmann's syndrome (KAL1), a developmental disorder characterized by anosmia and hypogonadism (Falardeau, Chung et al. 2008).

4.2.3 Transforming growth factor beta (TGF-β) roles and functions

The TGF-β superfamily of extracellular signaling molecules induces over 35 structurally related but functionally diverse proteins. These proteins function as extracellular ligands involved in numerous physiological processes.This superfamily has been classified into several subfamilies: The TGF-β subfamily (TGF-β1, TGF-β2,TGF-β3), the bone morphogenetic protein (BMP) subfamily, the growth and differentiation factor (GDF) subfamily, the activin/inhibin subfamily , the glial cell-drived neurotrophic factor (GDNF) subfamily and other members such as anti-mullerian hormone (AMH). Within the ovary, GDF9, BMP15, inhibin, activin and AMH are all expressed (Knight and Glister 2006). Function of TGF-β subfamily members vary widely from regulating folliculogenesis to regulating proliferation. GDF9 and BMP15 are expressed in

the oocyte from the preantral follicle growth. Studies in later stages of follicle development indicate an important positive role for granulosa cell-drived Activin, BMP2, BMP5 and BMP6, theca cell-drive BMP2, BMP7 and oocyte-drove BMP6 in promoting granulosa cell proliferation, follicle survival and prevention of premature luteinization and/or atresia. Secretion of TGF-β from theca cells increases LH receptor production by granulosa cells in response to FSH stimulation, whereas it inhibits androgen production by theca cells (Knight and Glister 2003).

Table 4. Other growth factors receptor, function and refrences

Growth factors	Receptor	Function	References
IGFs	IGF-IR(high affinity), IR (low affinity) and IGF-IIR(very low affinity)	IGF increase the expression of FSHR and LHR and stimulates the synthesis and secretion of E2, P4, testosteron, oxytocin, inhibin A, activin A and prostaglandins.	(Butler 2003, Quirk, Cowan et al. 2004). (Kwintkiewicz and Giudice 2009)
EGFs	EGFR/ErbB-1, HER2/ErbB-2, HER3/ErbB-3 HER4/ErbB-4	The main functions of EGF within the follicle are stimulating proliferation of GC , increasing P4 secretion, and controlling of the release of E2. In oocytes, EGF affects maturation and cumulus expantion, and inhibits apoptosis.	(Zhang, Berezov et al. 2007) (Jiang, Grenley et al. 2011
TGFβ	TGFβRI,TGFβRII,TGFβRIII	Regulate cellular activity. promoting granulosa cell proliferation, follicle survival and prevention of premature luteinization and/or atresia	(Knight and Glister 2006).

CHAPTER 2:

HYPOTHESIS AND OBJECTIVES

Hypothesis

FGF8 and FGF18 fibroblast growth factors have significant altering effect on ovine granulosa cell functions. They are activating specific intracellular signaling pathways that are involved either in apoptosis or proliferation. FGF8 and FGF18 are homologous factors which possess similar sequence homology, but we hypothesized they may interact with FGFRs differently leading to distinct effects, particularly on granulosa cell growth and induce proliferation following a short period of exposition.

Objectives

The objective of this study was to:

1. Perform bottom-up mass spectrometry–based proteomics for discovery and quantification of protein expression profile in granulosa cells following exposure to FGF8 and FGF18.
2. To determine which intracellular pathways were up- or downregulated following a time limited exposure of granulosa cells to FGF8 and FGF18, using bioinformatics and label-free proteomic approaches.

CHAPTER 3:
SHORT COMMUNICATION
(ARTICLE)

Differential Proteomic Analyses of Ovine Granulosa Cells Exposed to Fibroblast Growth Factor 8 or 18 Reveal Early Onset of Cell Growth and Survival via the Upregulation of Two Major Transcription Factors

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Abstract

The fibroblast growth factors (FGFs) are secreted molecules which function through the activation of a specific tyrosine kinase receptors, the FGF receptors that transduce the signal by activating several pathways. FGFs including FGF8 and FGF18 possess broad mitogenic and cell survival activities, and are involved in embryonic development, cell growth, tumor development and invasion. This study was performed to investigate the effects of FGF8 and FGF18 on ovine granulosa cells proteome. Using a bottom-up proteomic approach, mass spectrometry and a label-free quantitation method, results revealed following treatment with FGF8 or FGF18 for 30 minutes, an important shift toward upregulation for the entire granulosa cell proteome. Additionally, several proteins, including ATF1, STAT3, MAPK1, MAPK3, MAPK14, PLCG1, PLCG2, PKCA, PIK3CA, RAF1, GAB1 and BAG2 were significantly upregulated (> 1.5-fold; $p < 0.01$). Results are suggesting the activation of the MAPK/ERK pathway as expected. However, it is important to note that ATF1 and STAT3 are important transcription factors involved in cell growth, proliferation and survival and consequently can hamper or rescue the normal ovine reproductive system function.

Keywords: fibroblast growth factors, Proteomics, mass spectrometry, ovary, granulosa cells, transcription factors, proliferation

1. Introduction

The fibroblast growth factors (FGFs) are growth factors which have diverse biological activities including broad mitogenic and cell survival activities (Itoh and Ornitz 2004). FGFs constitute a large family of 22 distinct polypeptide growth factors varying in size from 17 to 34 kDa. They play a fundamental role in regulation of embryogenesis. More precisely, they are responsible for cell growth, differentiation, proliferation and cell migration. FGFs play a fundamental role in several stages of follicular development from preantral to preovulatory stage. Specifically, FGFs have been identified to regulate the initiation of primordial follicle growth, granulosa and theca cell proliferation, differentiation, angiogenesis and steroidogenes (Parrott, Vigne et al. 1994). It is presumed that FGF8 and FGF18 have similar receptor activation patterns leading to similar actions in ovine granulosa cells (Lavranos, Rodgers et al. 1994). A recent study proposed that FGF8 and FGF18 increase follicular health by increasing proliferation and suppressing cell differentiation (Buratini, Teixeira et al. 2005).

The FGF receptors (FGFRs) include four major receptors (FGFR1-4) that, like other RTK (receptor tyrosine kinase), are activated by specific ligands. FGFRs are composed of two or three Ig-like loops in the external domain, a transmembrane domain and a ligand-activated cytoplasmic tyrosine kinase domain (Ornitz, Xu et al. 1996, Furdui, Lew et al. 2006). The two membrane-proximal Ig loops (Ig-II and Ig-III) comprise the ligand binding domain, although alternative splicing of Ig-loop III can generate isoforms (III b and III c) with distinct ligand binding properties and tissue distributions (Ornitz and Itoh 2001). Binding of FGF and HSPG to the extracellular ligand domain of FGFR (FGFR1c, FGFR2c, FGFR3c, FGFR3b and FGFR4) induces receptor dimerization, activation and autophosphorylation of multiple tyrosine residues in the cytoplasmic domain of the receptor molecule (Ornitz and Marie 2002). A variety of signaling proteins are phosphorylated in response to FGF stimulation including Shc, PLCγ, STAT1, Gab1 and FRS2α leading to stimulation of intracellular signaling pathways that control cell proliferation, cell differentiation, cell migration, cell survival and cell shape (Sato and Nakamura 2004). The docking proteins FRS2α and FRS2β are major mediators of the

Ras/MAPK and PI-3 kinase/Akt signaling pathways as well as negative feedback mechanisms that fine-tune the signal that is initiated at the cell surface following FGFR stimulation (Eswarakumar, Lax et al. 2005). This pathway is important in granulosa cells (Dailey, Ambrosetti et al. 2005). Signal transducer and activator of transcription 3 (STAT3) was identified as a phospho-dependent partner for FGFRs. STAT3 has a key role in many cellular processes such as cell growth and apoptosis.

FGF8 and FGF18 are homologous factors which possess similar sequence homology, but we hypothesized they may interact to FGFRs differently leading to distinct effects, particularly on granulosa cell growth and induce proliferation following a short period of exposition. Thus, the objective of the present study was to compare and differentiate at the proteome level, the effects triggered by the activation of FGFRs (i.e. FGFR2 and FGFR3) with two ligands, FGF8 and FGF18, in ovine granulosa cell using a bottom-up proteomic, mass spectrometry, label-free quantitation and bioinformatics. The outcome of this study will help to foster a better understanding of the FGF signaling pathway outcomes.

2. Materials and Methods

All materials for cell culture were obtained from Life Technologies Inc. (Thermo Fisher Scientific, Burlington, ON, Canada) unless otherwise stated. Ovine granulosa cells were cultured in serum-free conditions that maintain estradiol secretion and responsiveness to FSH (Glister, Richards et al. 2005). Ovine ovaries were obtained from adult sheep's irrespective of stage of estrous cycle, at an abattoir and transported to the laboratory at 30˚C in phosphate-buffered saline (PBS) containing penicillin (100 µg/mL), streptomycin (100 µg/mL) and fungizone (1 µg/mL). Granulosa cells were harvested from follicles 1-2 mm diameter, and the cell suspension was filtered through a 150-mesh steel sieve (sigma-Aldrich Canada, Oakville ON). Cell viability was assessed by trypan blue dye exclusion. Cells were seeded into 24-well tissue culture plates (Sarstedt Inc., Newton, Nc) at a density of 0.5 million viable cells in 1 mL DMEM/F12 containing sodium bicarbonate (10 mmol/L), sodium selenite (4 ng/mL), bovine serum albumin (BSA) (0.1%; Sigma-Aldrich), penicillin (100 µg/mL), streptomycin (100 µg/mL), transferrin (2.5 µg/mL), nonessential amino acid mix (1.1 mmol/L), bovine insulin (10 ng/mL), androstenedione (10^{-7}M at start of culture and 10^{-6} M at each medium change) and

bovine FSH (10 ng/mL) starting on day 2; AFP5346D; National hormone and peptide program, Torrance, CA). Cultures were maintained at 37ºC in 5% CO2, 95% air for 5 days with 70% medium being replaced on days 2 and 4. To assess the effect of FGF8 and of FGF18 on intracellular pathway activation, cells were treated on day 5 of culture with 10 ng/mL recombinant human FGF8 and FGF18 (Pepro Tech) for 30 minutes, and cells lysed, and protein solubilized in RIPA buffer. All experiments were performed with three different pools of cells each collected on a different occasion. The samples were stored at -80˚C pending mass spectrometry analysis.

Proteins were extracted from cell samples and bottom-up proteomic analysis were performed. The total amount of protein in each sample was determined to use a standard Bradford assay. Briefly, a volume corresponding to 50 µg of proteins was used for each sample. Proteins were isolated using a precipitation procedure with a ratio 1:3 (v:v) of acetone. The samples were centrifuged at 9,000 g for 10 min. Then acetone was discarded, and protein pellet was dried for 20 minutes in a vacuum centrifuge set at 60˚C. The protein pellet was dissolved in 100 µL of 50 mM ammonium bicarbonate (pH 8) and the solution was sonicated for 60 minutes at maximum intensity to improve protein dissolution yield. Reduction and alkylation were performed as previously described [Ruiz et al. 2015] and then, 2 µg of proteomic-grade trypsin was added, and the reaction was performed at 40˚C for 24h. The protein digestion was quenched by adding 10 µL of a 2% TFA solution. Samples were centrifuged at 12 000 g for 10 min and the supernatants were transferred into injection vials for analysis.

The HPLC system was a Thermo Scientific UltiMate 3000 Rapid Separation UHPLC system (San Jose, CA, USA). The chromatography was achieved using a gradient mobile phase along with a microbore column Thermo Biobasic C8 100 × 1 mm, with a particle size of 5 µm. The initial mobile phase condition consisted of acetonitrile and water (both fortified with 0.1% of formic acid) at a ratio of 5:95. From 0 to 1 minute, the ratio was maintained at 5:95. From 1 to 61 minutes, a linear gradient was applied up to a ratio of 50:50 and maintained for 2 minutes. The mobile phase composition ratio was reverted at the initial conditions and the column was allowed to re-equilibrate for 14 minutes for a total run time of 77 minutes. The flow rate was fixed at 75 µL/min and 2 µL of samples were injected. A Thermo Scientific Q Exactive Orbitrap Mass Spectrometer (San Jose, CA, USA) was interfaced with a Thermo Scientific UltiMate

3000 Rapid Separation UHPLC system using a pneumatic assisted heated electrospray ion source. MS detection was performed in positive ion mode and operating in scan mode at high-resolution, and accurate-mass (HRAM). Nitrogen was used for sheath and auxiliary gases and they were set at 10 and 5 arbitrary units. The ESI voltage was set to 4000 V and the ion transfer tube temperature was set to 300°C. The default scan range was set to m/z 400-1500. Data was acquired at a resolving power of 140,000 (FWHM) using automatic gain control targets of $3.0x10^6$ and maximum ion injection time of 200 msec. Additionally, MS data were acquired using a data-dependent top-10 method to dynamically choose the most abundant precursor ions from the survey scans (400–1500 Da) and generate MS/MS spectra. Instrument calibration was performed prior to all analysis and mass accuracy was notably below 1 ppm using Thermo Pierce calibration solution and automated instrument protocol.

Database surveys were performed using Proteome Discoverer software (version 2.1) with Uniprot ovine protein database (extracted FASTA file). Mass tolerance of precursor and fragment (i.e. typically b and y) were set at 5 ppm and 10 ppm, respectively. Phosphorylation at Y and T amino acids was set as a variable post translational modification. Quantification was based on MS^1 ion intensity and peptide identification was based on precursor ion (MS^1) and at least three characteristic (MS^2). Label-free MS^1 quantification of peptide/protein via peak intensity was performed using SIEVE (version 2.1), a label-free differential expression software that aligns the MS spectra over time from different data sets and then determines structures in the data (m/z and retention time pairs) that differ. These differences were examined using statistical methods (e.g. p-value and standard deviation) and then sorted based on significance using the peak intensity values obtained from the data of each biological replicate. The following parameters were set to align the retention time and generate the frames needed for abundance calculations. Alignment Parameters; Alignment Bypass = False, Correlation Bin Width = 1, RT Limits for Alignment = True, Tile size = 300, Max RT Shift = 0.2, m/z Min = 400, m/z Max = 1,500, Frame time Width (min) = 2.5 minutes, Frame m/z width = 10 ppm, Retention Time Start = 2.0 min, Retention Time Stop = 65 min, Peak Intensity threshold = 100,000. Significance was calculated within SIEVE using a standard t-test and results were filtered using the identification criteria stated above. Statistical significance was set at a p-value < 0.01. Interactomic analyzes were performed using Genemania and STRING interfaces and databases.

3. Results and discussion

Detected tryptic peptides were used to identify proteins (i.e. MS^1 and MS^2 levels) and quantified (i.e. MS^1 level) using a label-free proteomic approach. Volcano plot was used to analyze differential protein expression and compare control samples with ovine granulosa cell exposed to 10 ng/mL of FGF8 or FGF18 for 30 minutes. Figure 1 exhibits a Volcano plot revealing differentially expressed proteins between control and FGF8 (Figure 1A) as well as control versus FGF18 (Figures 1B). As displayed, the exposition of ovine granulosa cells to FGF8 or FGF18 has led to a significant up-regulation of numerous proteins and very few were down-regulated. Moreover, very few proteins were unaffected by either FGF8 or FGF18. This observation is coherent with an early onset of cell growth and proliferation, an expected outcome since FGFR signaling pathways are associated with both. Additionally, despite it was not statistically significant, we observed a more important up-regulation following the exposition to FGF18 compared to FGF8. This observation is compatible with our initial hypothesis. FGF8 and FGF18 are homologous growth factors which possess similar sequence homology, but they appear to interact with FGFRs differently leading to a more pronounce growth or proliferation effect that may lead to a distinct outcome for granulosa cells.

Protein abundance were sorted based on spectral abundances and Table 1 includes the most abundant proteins observed and validated based on spectral libraries and *in silico* bottom-up proteomic analyses (e.g. Proteome Discoverer – SEQUEST). A total of 32 up-regulated proteins (i.e. fold change > 1.5 for FGF8 or FGF18) were identified as shown in Table 1. Interestingly, two very important transcription factors were identified, STAT3 and ATF1. STAT3 is a member of the STAT family that was identified as a DNA-binding factor (Wegenka, Buschmann et al. 1993; Akira, Nishio et al. 1994; Lutticken, Wegenka et al. 1994) and is expressed early during post-implantation in most tissues. Interestingly, $STAT3^{-/-}$ mice are characterized by embryonic lethality. STAT3 is involved in promoting cell growth and constitutively active and potentiate tumorigenesis (Bromberg and Darnell 2000; Yu, Pardoll et al. 2009). Pro-oncogenic STAT3 activity is linked with the gene expression that is known to promote proliferation and inhibit apoptosis (Regis, Pensa et al. 2008). Several serine kinases have been reported to be involved in the serine phosphorylation of STATs, including MAPK14 and MAPK1/3 also up-regulated

following the exposition of granulosa cell to FGF8 and FGF18 for 30 minutes. With the advances in proteomic science, STAT3 was identified as an important molecule that plays numerous roles in cells. It has a two-fold role as a mediator of signaling and regulator of gene expression. Furthermore, STAT3 has been widely described as an oncogene correlated with tumor progression and up-regulated in many tumor specimens. Consequently, STAT3 signaling pathway could be considered as a promising therapeutic target. As shown in Table 1 and Figure 2, many members of the MAPK/ERK pathway were significantly up-regulated following the exposition of granulosa cells to FGF8 and FGF18 for 30 minutes. Growth factors and mitogens use the MAPK/ERK pathway signaling cascade to signal the regulation of gene expression and prevent apoptosis. Some constituents of these pathways are mutated or abnormally expressed in cancer. However, it may be advantageous to induce MAPK/ERK pathway expression to promote cell cycle arrest to stop deregulated cell proliferation. Activating transcription factor 1 (ATF1) was significantly up-regulated following the exposition of granulosa cells to FGF8 and FGF18 for 30 minutes. ATF1 and RAS proteins were found significantly elevated in various tumors and were recently identified as potential clinical diagnostic biomarkers (Shi et al. 2017). Also, an important aspect is that ATF1 is up-regulated upon stress as part of a cell adaptation mechanism. ATF1 and CREB interact and are important for cell survival, particularly during early development. The extensive up-regulation of ATF1 could be a consequence of the onset of STAT3 promoting cell growth.

Analysis of the STAT3 and ATF1 interactome (Genemania interface and database) shown in Figure 3, outline important genetic interactions and co-expression (Figure 3A and 3C) for all the abundant proteins observed presented in Table 1 and Figure 2. There is a strong genetic association between STAT3 and ATF1 but a minor physical or pathway association. This observation is also validated with a STRING analysis (i.e. predicted protein–protein interactions) in Figure 3D. This is coherent with our hypothesis that ATF1 might counterbalance the STAT3 induced cell growth, proliferation and potentiation of tumorigenesis using an independent pathway. The interactomic analyzes performed with Genemania and STRING presented in Figure 3, confirm a high degree of genetic and physical interactions between MAPK/ERK pathway signaling cascade with STAT3 and ATF1 coherent with symmetrical up-

regulation observed following the exposition of granulosa cell to FGF8 and FGF18 for 30 minutes.

Conclusion

In summary, in ovine granulosa cells, FGF8 and FGF18 induced a significant up-regulation of numerous proteins associated with the MAPK/ERK pathway. More importantly, two important transcription factors were significantly up-regulated, STAT3 and ATF1, both interacting strongly with the MAPK/ERK pathway. Interestingly, STAT3 is involved in promoting cell growth and proliferation and constitutively active STAT3 is able to potentiate tumorigenesis.

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TABLE 1. Proteins whose level of phosphorylation was increased in response to FGF8 and FGF18 in ovine ovarian granulosa cells

	Accession	FGF8		FGF18	
Protein	No	Fold change[1]	SD	Fold change[1]	SD
CAMK2A	Q9UQM7	1.41	0.056	1.56	0.067
YWHAB	P31946	1.23	0.042	1.58	0.065
GRB10	Q13322	1.29	0.030	1.59	0.040
TRADD	Q15628	1.25	0.035	1.60	0.040
EPHA5	P54756	1.40	0.086	1.62	0.086
PLCG2	P16885	1.35	0.042	1.64	0.051
IRF2BP1	Q8IU81	1.48	0.042	1.64	0.062
PIK3CA	P42336	1.40	0.030	1.65	0.044
YWHAE	P6225	1.58	0.025	1.67	0.046
STAT3	P40763	1.54	0.058	1.67	0.065
IGF1	Q00997	1.46	0.04	1.69	0.054
MDM2	Q00987	1.47	0.075	1.71	0.076
PLCG1	P19147	1.68	0.182	1.71	0.206
ATK1	O00139	1.57	0.080	1.71	0.098
KRAS	P01116	1.44	0.098	1.71	0.160
MAPK14	Q16539	1.28	0.055	1.72	0.081
MAPK3	P27361	1.79	0.141	1.76	0.074
MAP2K1	Q02750	1.29	0.291	1.77	0.348
HRAS	P01112	1.35	0.162	1.78	0.229
MAPK1	P28482	1.88	0.037	2.01	0.043
PGRMC1	O00264	1.85	0.062	2.02	0.118
CTSA	P10619	1.68	0.081	2.04	0.121
GPS2	Q13227	1.45	0.138	2.08	0.203
XDH	P47989	1.68	0.086	2.12	0.102
RASGRP3	Q81V61	1.58	0.177	2.32	0.275
GAB1	Q13480	1.79	0.263	2.67	0.371
RAF1	P04049	2.07	0.510	2.75	0.645
BAG2	O95816	2.03	0.881	3.07	1.069
SNX3	O60493	1.78	0.408	3.09	0.434
SF3A3	Q12874	3.47	1.316	4.39	1.648
PKCA	P17252	3.52	1.581	4.44	1.764
ATF1	P18846	6.11	2.983	8.90	3.793

[1] $p < 0.01$ or better

List of Figures

Figure 1.

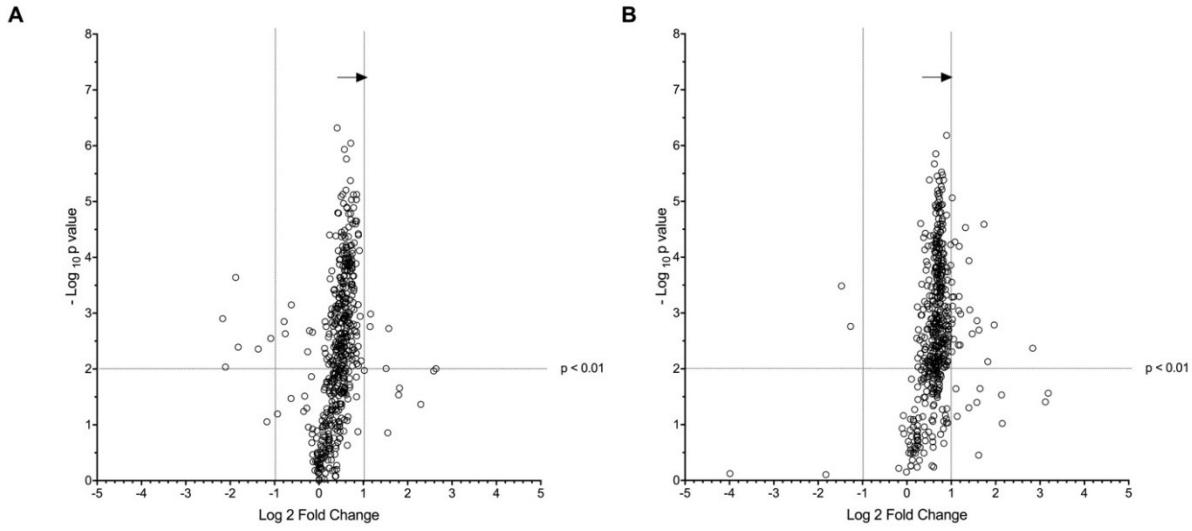
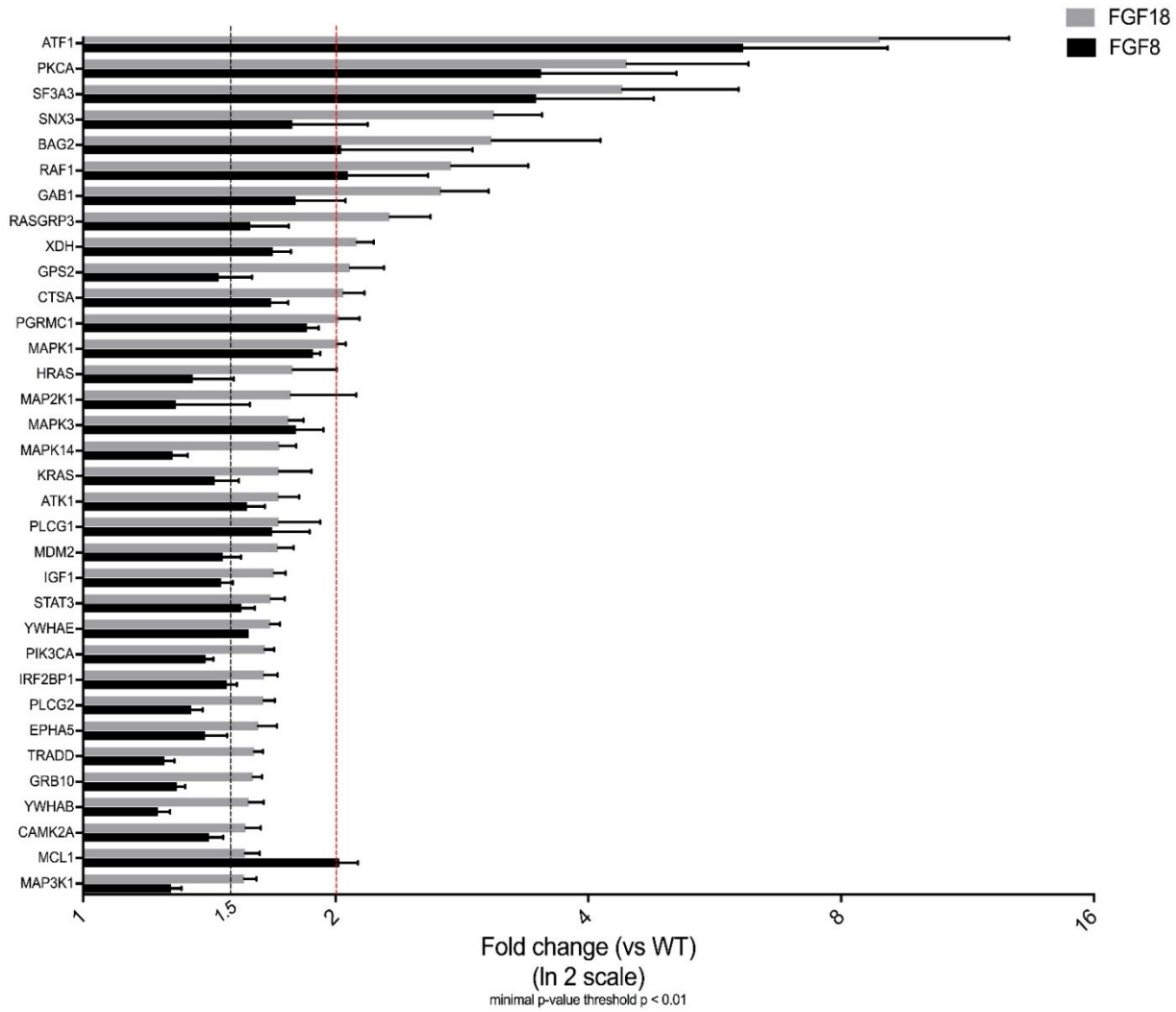


Figure 2.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FGF8
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PKCA
SF3A3
SNX3
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RAF1
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RASGRP3
XDH
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MAPK1
HRAS
MAP2K1
MAPK3
MAPK14
KRAS
ATK1
PLCG1
MDM2
IGF1
STAT3
YWHAE
PIK3CA
IRF2BP1
PLCG2
EPHA5
TRADD
GRB10
YWHAB
CAMK2A
MCL1
MAP3K1
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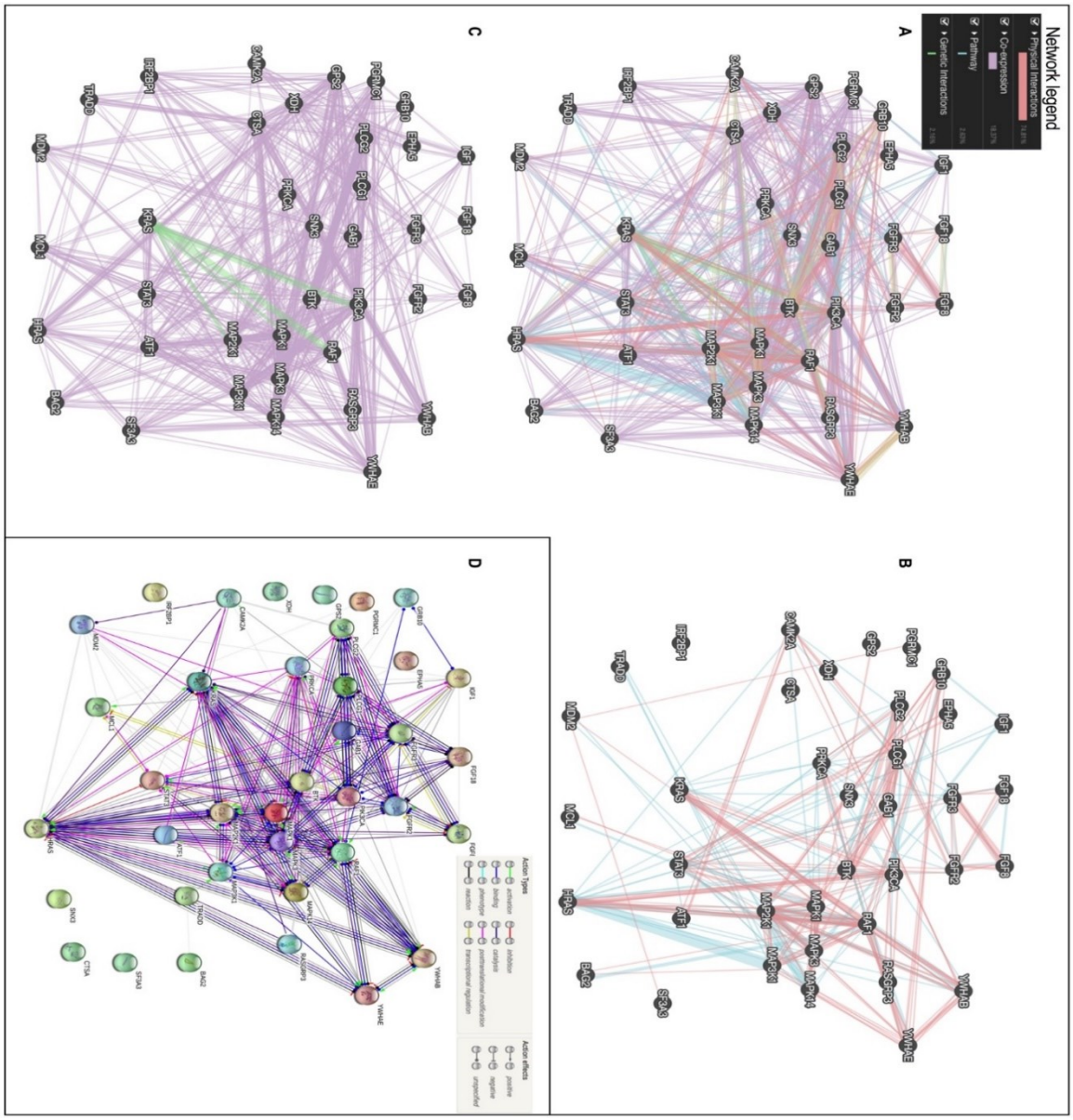
16
Fold change (vs WT)
(ln 2 scale)
minimal p-value threshold p < 0.01

Figure 3.


Network legend
Physical Interactions
Co-expression
Pathway
Genetic Interactions
A
B
C
D
Action Types
Action effects

CHAPTER 4:

GENERAL DISCUSSION

1. Summary of the results

Proteomic analysis using a "bottom-up" strategy was performed following a 30 minutes FGF8 or FGF18 exposition to granulosa cells. Detected tryptic peptides were used to identify proteins (i.e. MS^1 and MS^2 levels) and quantified (i.e. MS^1 level) using a label-free proteomic approach. Differential analyses and comparison was performed using a Volcano plot. The Volcano plots revealed differentially expressed proteins between control and FGF8 as well as control versus FGF18. As shown, the exposition of ovine granulosa cells to FGF8 or FGF18 led to a significant up-regulation of several proteins and very few were down-regulated. Furthermore, only few proteins were unaffected by either FGF8 or FGF18. Despite it was not statistically significant ($p > 0.05$), we detected a more important trend toward up-regulation following the exposition to FGF18 compared to FGF8. This observation is compatible with our initial hypothesis. FGF8 and FGF18 are homologous growth factors which possess similar sequence homology, but they appear to interact with FGFRs differently leading to a more pronounce growth or proliferation effect that may lead to a distinct outcome for granulosa cells. Protein abundance were sorted based on spectral abundances and we focused only on the most abundant proteins observed (i.e. Threshold was $5x10^5$ cps) and validated based on spectral libraries and *in silico* bottom-up proteomic analyses (e.g. Proteome Discoverer – SEQUEST). A total of 32 up-regulated proteins (i.e. fold change > 1.5 for FGF8 or FGF18) were identified. Within these 32 up-regulated proteins, the most important were ATF1, STAT3, MAPK1, MAPK3, MAPK14, PLCG1, PLCG2, PKCA, PIK3CA, RAF1, GAB1 and BAG2. These results are suggesting the activation of the MAPK/ERK pathway as expected. It is also notable that two important transcription factors were significantly up-regulated, STAT3 and ATF1. Both are strongly interacting with the MAPK/ERK pathway. Interestingly, STAT3 is involved in promoting cell growth and proliferation and constitutively active STAT3 is able to potentiate tumorigenesis.

2. Relevance of the observation

We observed a significant up-regulation of MAPK1 (e.g. ERK2), MAPK3 (e.g. ERK1) and MAPK14 (e.g. p38) following the exposition of ovine granulosa cell to FGF8 and FGF18. The activated protein kinases are associated to mitogenesis and cell survival. We believe that an up-regulation of MAPK1, MAPK3 and MAPK14 will lead to a significant increase of

phosphorylated MAPK1, MAPK3 and MAPK14 directing cellular responses toward cell growth and proliferation. This observation is coherent with our hypothesis leading to an early onset of cell growth and proliferation, an expected outcome since FGFR signaling pathways are associated with both. Additionally, despite it was not statistically significant, we observed a more important protein up-regulation following the exposition to FGF18 compared to FGF8. This observation was also compatible with our initial hypothesis. FGF8 and FGF18 are homologous growth factors which possess similar sequence homology, but they appear to interact with FGFRs differently leading to a more pronounce growth or proliferation effect that may lead to a distinct outcome for granulosa cells. We can hypothesize that, hyperplasia of ovine granulosa cells within individual retained follicles in *in vivo* situation could be the consequence of FGF8 or FGF18 activation of the FGFR pathway with a more significant effect for FGF18. Interestingly, concomitant endometrial pathology in form of hyperplasia is a common finding in granulosa cell tumor. Moreover, elevated estrogens are observed during hyperplasia of granulosa cells and it is suspected that estradiol play an important role in the regulation of FGFR induction (Vernon and Spicer 1994). It has been demonstrated that overexpression of FGF8 or FGF18 was related to increase tumor growth and angiogenesis (Korc and Friesel 2009).

More generally proteomic analysis using mass spectrometry and bioinformatics demonstrate that, several intracellular signaling cascades might be activated or deactivated following a limited exposure of ovine granulosa cells to FGF8 and FGF18. However, without accurate quantification of mitogen-activated protein kinase specific phosphorylated peptides, it is hazardous to be conclusive. We can only mention that an up-regulation of mitogen-activated protein kinases will most likely lead to an increase in concentration of phosphorylated mitogen-activated protein kinases leading to cascades of intracellular signaling. MAPK1 is a Ser/Thr kinase which is phosphorylated by MAP2K1/MEK and MAP2K2/MEK2 on thr-185 and tyr-187 in response to external stimuli, and mediates many biological functions such as cell growth, survival and differentiation (Johnson and Lapadat 2002) and it is a critical component of the Ras-Raf-MEK-ERK signal transduction cascade. MAPK1 is a molecule that is significantly upregulated by both FGF8 and FGF18 in ovine ovarian granulosa cells. The ERK (e.g. MAPK1 and MAPK3) cascade is highly upregulated in human cancers, and is typically activated by growth factor stimulation of the cell surface receptor tyrosine kinase (RTKs) and other signaling

molecules with known oncogenic potential (Wilhelm, Carter et al. 2004).The RTKs span the cell membrane, and the intracellular catalytic domains possess tyrosine kinase activity, catalyzing the transfer of the γ-phosphate of ATP to the OH groups of tyrosine residues (Pearson, Robinson et al. 2001). MAPK1 and MAPK3 are both expressed in most, if not all, mammalian tissues, however, MAPK1 levels are generally higher than MAPK3. Knock-out studies in mice showed that MAPK3 has been found to specifically regulate thymocyte maturation (Fischer, Katayama et al. 2005). Downstream, activated MAPK1/3 regulates growth factor-responsive targets in the cytosol and translocate to the nucleus where it phosphorylates several transcription factors regulating gene expression (McCubrey, Steelman et al. 2007). MAPK1/3 indirectly regulates translation by inducing tRNA and rRNA synthesis. Both regulate transcription indirectly by phosphorylating the 90 kDa ribosomal protein S6 kinases (RSKs), a family of broadly expressed serine/threonine kinases activated in response to mitogenic stimuli including growth factors and tumor-promoting phorbol esters. Active RSKs seems to play an important role in transcriptional regulation by translocating to the nucleus and phosphorylating factors such as c-*fos* at Ser362, serum response factor (SRF) at Ser103, and cyclic AMP response element-binding protein (CREB) at Ser133 (Dhillon, Hagan et al. 2007). The MAPK1 is deactivated by DUSP3 (dual-specific phosphatase 3), DUSP6 and DUSP9 which are a sub-class of the ubiquitous protein tyrosine phosphatases (PTPases) that are uniquely able to hydrolyze the phosphate ester bond on both a tyrosine residue and either a serine or threonine residue located on the same protein. Cell-division cycle 25 (CDC25) DUSPases have been shown attractive targets for novel anticancer agents, as they have a crucial role in controlling cell-cycle progression and genetic instability (Lyon, Ducruet et al. 2002).

MAPK14 (p38) is an essential component of MAPK family and is involved in cellular response to pro-inflammatory cytokines. Depending on the organ studied, it can act as pro- or anti-apoptotic factor (Kim, Sano et al. 2008, Gao, Smit et al. 2013). It is also highly expressed in many malignant tissues. As shown by our results, MAPK14 expression is significantly increase in response to FGF8 and FGF18. It can potentially lead to an increase of phosphorylated MAPK14 concentration in ovine granulosa cells. Phosphorylated MAPK14 activates numerous downstream substrates, including MAPK-activated protein kinase-2 and 3 (MAPKAPK-2 or 3) and MSK1/2 (mitogen and stress-activated kinase 1/2) which in turn phosphorylates heat shock

protein 27 (HSP27) and cAMP-response element binding protein transcription factor respectively. These kinases in turn activate other transcription factors, including transcription factor 2, ELK, CHOP/GADD153 and myocyte enhancer factor 2.

Interestingly, our results shown a very important up-regulation of accelerating transcription factor 1 (ATF1). Functionally, ATF1 binds the cAMP response element (CRE). ATF1 is associated to cell growth, survival and proliferation. It is phosphorylated at serine 63 on its kinase-inducible domain by serine/threonine kinase, cAMP proteins kinase A, calmodulin dependent protein kinase I/II, mitogen and stress activated protein kinase and cyclin-dependent kinase 3. This protein is a cancer related protein and is involved in angiomatoid fibrous histocytoma and clear cell sarcoma (Hallor, Mertens et al. 2005). ATF1 is also involved in soft tissue cancers, melanoma, bone, skin, lung and salivary gland cancer (Wang, Mayordomo et al. 2009). ATF1 and RAS proteins were found significantly elevated in various tumors and were recently identified as potential clinical diagnostic biomarkers (Shi et al. 2017). Also, an important aspect is that ATF1 is up-regulated upon stress as part of a cell adaptation mechanism. ATF1 and CREB interacts and are important for cell survival, particularly during early development (Bleckmann, Blendy et al. 2002). The extensive up-regulation of ATF1 could be a consequence of the onset of STAT3 promoting cell growth.

Signal Transducers and Activators of Transcription 3 (STAT3) is an important transcription factor regularly associated with cancer. STAT3 was also significantly up-regulated following the exposition of ovine granulosa cells to FGF8 and FGF18. In response to cytokines and growth factors, STAT family members are phosphorylated by serine kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. Several serine kinases have been reported to be involved in the serine phosphorylation of STATs, including MAPK14 and MAPK1/3 also up-regulated following the exposition of granulosa cells to FGF8 and FGF18 for 30 minutes. The up-regulation of MAPK1/3 may lead to further activation of the STAT3 pathway. Recent studies discovered constitutive activation of STAT3 in a wide variety of tumors, including hematological malignancies as well as diverse solid tumors including breast, lung, gastric, colorectal, ovarian and prostate cancers (Buettner, Mora et al. 2002, Kusaba, Nakayama et al. 2005). Transcriptomic and proteomic data shown a strong relationship suggesting that aberrant STAT3

signaling to promote the initiation and progression of several cancer types by inhibiting apoptosis or inducing cell proliferation, angiogenesis, invasion, and metastasis. Interestingly, the inhibition of STAT3 activation mechanism results in the induction of apoptosis in tumor cells, and accordingly its pharmacological modulation using several therapeutic strategies were used effectively to suppress the proliferation of various cancer cells in culture and tumorigenicity using *in vivo* models. It is promising, but there is no treatment yet available for patients. Analysis of the STAT3 and ATF1 interactome (Genemania interface and database) outlines important genetic interactions and co-expression for all the abundant up-regulatedm proteins observed. Moreover, a strong genetic association between STAT3 and ATF1 but a minor physical or pathway association was identified. This observation is also validated with a STRING analysis (i.e. predicted protein–protein interactions) where no action types were identified between STAT3 and ATF1. This is coherent with our hypothesis that ATF1 might counterbalance the STAT3 induced cell growth, proliferation and potentiation of tumorigenesis using an independent pathway. The interactomic analyzes performed with Genemania and STRING, confirm a high degree of genetic and physical interactions between MAPK/ERK pathway signaling cascade with STAT3 and ATF1 coherent with symmetrical up-regulation observed following the exposition of granulosa cells to FGF8 and FGF18 for 30 minutes. There are clear evidence supporting critical roles of STAT3 in oncogenesis. A better understanding of the ATF1 and STAT3 interaction may lead to the development of innovative granulosa cell tumors therapies based on mechanistic understanding of ATF1 and STAT3 signaling cascades.

PI3K was also up-regulated following FGF8 and FGF18 granulosa cell exposition. Interestingly, constitutive activation of PI3K in oocyte induces ovarian granulosa cell tumors (Reddy, Liu et al. 2008). The PI3K–TOR and STAT3 signaling pathways are two distinct regulatory networks. The recent discovery of a functional association between these pathways is potentially important and may also lead to a better understanding of PI3K- and STAT3-driven oncogenic mechanisms.

3. Implication of the results

In ovine granulosa cells, FGF8 and FGF18 induced a significant up-regulation of numerous proteins associated with the MAPK/ERK pathway but more importantly, two important transcription factors were significantly up-regulated, STAT3 and ATF1. Both are strongly interacting with the MAPK/ERK pathway. STAT3 is a transcription activator and an oncogene that is tightly regulated under normal physiological conditions. As mention before, recent data suggest that STAT3 is activated in several cancer types, with a fundamental role in tumor onset and progression. STAT3 is regulated by canonical cytokines and growth factors, G-protein-coupled receptors, cadherin engagement, Toll-like receptors (TLRs), and microRNA (miRNA). Despite the presence of various regulators and essential biological functions in cancer, actually, there are no effective therapeutics available for inhibiting STAT3 and acquiring potent antitumor effects in the clinic. However, *in vitro* data has demonstrated that STAT3 pathway can be a future therapeutic target.

Interestingly, a higher ATF1 expression appears to be a predictor of a favorable outcome for the overall survival and progress of a few cancer types. ATF1 might counterbalance the STAT3 induced cell growth, proliferation and potentiation of tumorigenesis using an independent pathway. Thus, ATF1 could be a good efficient biomarker specifically used to monitor treatment targeting STAT3 pathway. Clearly, these observations are key to foster a better understanding of the complex roles of STAT3 in cancer and potentially develop new medicines to achieve optimal therapeutic effects.

4. Limitation

This study examined the effects of a single dose at a single time-point of exposure. Future experiments should be aimed at establishing a dose response to better characterized differential effects of FGF8 and FGF18 on granulosa cell proteome. Moreover, accurate quantification of specific phosphopeptides will allow to determine a time course of phosphorylation events to better understand how MAPKs are activated to signals intracellular responses. The data generated in this study quantified the expression of the entire proteome and was not able to properly quantified phosphorylation at specific amino acids. At this point, we can only suspect

that an up-regulation of MAPK1, MAPK3 and MAPK14 will result in an increase of the intracellular concentration of phosphorylated MAPK1, MAPK3 and MAPK14 signaling specific intracellular responses. Thus, leading to an up-regulation of two important transcription factors, STAT3 and ATF1. Our data is insufficient to adequately validate this, and more experiments will be required.

CONCLUSION

Conclusion

In summary, in ovine granulosa cells, FGF8 and FGF18 induced a significant up-regulation of numerous proteins associated with the MAPK/ERK pathway but more importantly, two important transcription factors were significantly up-regulated, STAT3 and ATF1. Both are strongly interacting with the MAPK/ERK pathway. Interestingly, STAT3 is involved in promoting cell growth and proliferation and constitutively active STAT3 can potentiate tumorigenesis. The impact of STAT3 on ovaries health is not well-defined but activation of Ras/Raf/MEK/MAPK1/MAPK3 signaling cascades causing the activation of STAT3 pathways can lead to cell growth and proliferation, and may have an impact on fertilization and follicle health. However, the functional biology of the putative interactions of STAT3 needs additional investigations. Further research needs to better define specific outcomes and how it may affect the follicle dominance and ovulation. This may allow the selection of potential targets to be pharmacologically manipulated with the objective of improving fertility efficiency and ability of embryos to develop, to implant and generate a healthy offspring. These results may provide new insights to foster better knowledge of factors affecting the oocyte competence embryo quality and potentially ovarian disorders in animal and human.

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